

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 55 ART. 2 PAGES 37-344

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THE CHICK EMBRYO IN BIOLOGICAL RESEARCH

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NEW YORK

PUBLISHED BY THE ACADEMY

August 8, 1952

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VOLUME 55, ARTICLE 2. PAGES 37-344

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ROY WALDO MINER

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Conference Chairmen: M. E. RAWLES AND D. A. KARNOFSKY

Consulting Editor: D. A. KARNOFSKY

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* This series of papers is the result of a Conference on *The Chick Embryo in Biological Research*, held by the Section of Biology of The New York Academy of Sciences, December 7 and 8, 1951.
The Conference and this resulting publication have been made possible by a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

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INTRODUCTION: BASIC SCIENTIFIC RESEARCH IN RELATION TO ITS APPLICATIONS

By E. D. Goldsmith

New York University College of Dentistry and the Office of Naval Research

The conviction that the solution of problems in one field of science is facilitated by the coordinated thoughts, experiences and skills of many disciplines has become irresistible. As each day and hour, however, bring new achievements, emphasis is often shifted from the basic findings to the applications made therefrom. These applications are, of course, exciting, and in them one readily recognizes some utility. They lend themselves to exploitation and, in this exploitation, the basic contributions sometimes tend to be forgotten. Fortunately, thanks to the wisdom and efforts of a number of our eminent scientists, to several government agencies and to a number of our foundations, there is ample promise that a proper balance will be maintained.

The conference on which this monograph is based ably illustrates this thesis, thanks to the guiding hands of Dr. Mary E. Rawles and Dr. David A. Karnofsky. For years, students have concerned themselves with the basic biological, chemical and physical aspects of the chick egg. There was no thought of application. To some, it was a possible means of ascertaining whether the mechanist or the vitalist was correct; and to others, it provided the sheer joy of discovering what this structure, which contained all the potentialities of the adult chick, would do under controlled experimental conditions. Whatever the reasons may have been, a mass of data was accumulated; and it was to this fundamental knowledge so laboriously obtained and to the important applications which it has sparked that this monograph is devoted. It is hoped that this monograph will serve as a source book to provide researchers with information essential for the proper use of an organism as a research tool. A study of the excellent papers and their bibliographies will reveal that this objective has been attained. What additional investigations this monograph will catalyze we can but conjecture. That it will be productive can best be expressed in words of the late Dr. Walter B. Cannon, "Precisely because men are gathered together and talk freely about their problems fresh thoughts emerge and are elaborated."

METABOLISM OF THE EARLY EMBRYO

By Nelson T. Spratt, Jr.

University of Minnesota, Minneapolis, Minnesota

The primary purpose of this review is to present a summary and evaluation of our knowledge of the metabolic processes in the chick embryo during the first four days of its development after laying of the egg. Since this is a period of relatively rapid and profound developmental activity, during which the ground plans of many organ systems become visible, an understanding of the associated biochemical activities is of particular interest and importance.

The data to be reviewed have been selected, as far as possible, on the basis of their contribution to our understanding of the chemical reactions underlying developmental processes, not merely on the basis of their contribution to our knowledge of the biochemistry of the early embryo. This distinction is in accord with the aim of modern chemical embryology and probably is an important one if any real progress is to be made. In other words, the biochemical or metabolic properties to be considered are primarily those that refer to or underlie particular developmental processes, events or changes between embryonic stages of development.

Respiratory Metabolism at Different Stages

Early studies of oxygen consumption of the chick embryo (Bohr and Haselbalch, 1900; Murray, 1926; Needham, 1932a, b) were unfortunately carried out on the basis of wet weights of the embryos. Since there is no standardized or accurate procedure for obtaining the wet weight of an embryo prior to four days' incubation age, comparison of the results obtained either by different workers or at different stages of development is of little value to us. In more recent studies by Philips (1941, 1942) and Romanoff (1943), the more accurate methods used (measurement of mm^3 of oxygen consumed per mg. dry weight or per mg. nitrogen per hour = QO_2) provide us with some valuable and interesting facts.

(A) *The First Day.* The earlier studies of Philips (1941) show that the QO_2 increases by about 100 per cent during the first four hours of incubation. Thus there is an apparent (but see below) activation of respiratory metabolism which is correlated with the initiation of developmental processes. This observation is interesting since the marked difference in QO_2 between the unincubated and four-hour blastoderm has a parallel in the capacity of these embryos to undergo further development *in vitro* on either yolk, albumen or glucose-Ringer media (Spratt, 1950c). Explanted, unincubated blastoderms rarely undergo further growth, morphogenesis, or cellular differentiation, in contrast to those incubated about four or five hours prior to explantation, which frequently form an embryonic axis consisting of a brain, spinal cord, somites, notochord and heart.

Later studies of Philips (1942) show, however, that the QO_2 of the area pellucida alone continues to rise until about the twentieth hour of develop-

ment. Although this is the period of morphogenetic movements associated with mesoderm invagination and primitive streak (blastopore) formation, the increase is, according to Philips, apparently due to the increase in mass of respiring protoplasm which accompanies the utilization of intracellular yolk (since the Kjeldahl nitrogen does not increase significantly during this period) and thus is not a reflection of any increase in developmental activities. The apparently more rapid rise in the Q_{O_2} during the first seven hours of incubation (Philips, 1942, FIGURE 3) may, even if significant, only represent a greater rate of yolk utilization compared with the eight to twenty hour period (*cf.* Gregg and Lovtrup, 1950). The use of desoxyribose nucleic acid content (an approximate measure of the amount of nuclear material might be a more accurate reference component on which to base the Q_{O_2}).

(B) *The Second to the Seventh Day.* Following this initial rise in Q_{O_2} the data of Philips (1941, 1942) and Romanoff (1943) clearly indicate that the level of the rate of oxygen consumption of the embryo remains relatively constant through the seventh day of incubation. Here, in contrast with earlier stages, practically all the nitrogen on which the Q_{O_2} is based has been incorporated into active cellular materials.

It seems apparent from the data that there is no significant change in respiratory metabolism of the chick embryo during its first week of incubation. This observation is not necessarily surprising if one admits: (1) that there is no *a priori* reason for assuming either an increase or decrease during this period in over-all developmental activity which would be reflected in respiratory metabolism; or (2) that if there were a true increase or decrease in total developmental activity, it would not necessarily result in a change in oxygen consumption. Undoubtedly there are changes in activity of particular developmental processes (morphogenesis, cellular differentiation) and many of these seem to depend to a varying degree upon the available oxygen supply (Spratt, 1950a), but such individual differences in oxygen requirements would not necessarily change the total oxygen consumption of the embryo.

One might raise the question in this connection as to whether there are any changes in actual or potential respiratory enzyme activities during early development. Albaum and Worley (1942) reported that cytochrome oxidase does not appear until the fourth day of incubation. Moog (1943), however, was able to show that activity of what is presumably the same enzyme (indophenol oxidase) is present in head-process blastoderms, increases up to the middle of the second day, and is maintained at that level through the fourth day. Quantitative work in progress (Spratt, 1951c) indicates at the present time that the reducing enzyme activities of homogenates of one- to eight-day embryos do not change significantly.

Respiratory Metabolism of Different Embryonic Regions

Embryologists have known for many years that different regions of the chick blastoderm (15-30 hours' incubation) exhibit rather striking differences in developmental activity (*cf.* Dursy, 1866). For example, the node at the anterior end of the primitive streak (= dorsal lip of the blastopore) is a lo-

calized center of activity from which such axial structures as the notochord, neural tube, somite mesoderm, *etc.* are progressively formed as the node moves posteriorly (Balfour, 1881, Spratt, 1947a, Gaertner, 1949). The fore-brain is a secondary center of activity. Would these differences in visible developmental activity be expressed as quantitative respiratory differences?

By means of the Cartesian-diver microrespirometer, Philips (1942) measured the rate of oxygen consumption of various isolated regions (including the node region) of the head-process blastoderm. No regional differences of any significance could be found in the rate of cellular oxidations per mg. nitrogen (Q_{O_2}). Again, this result is not necessarily surprising since visible differences in developmental activity may not be expressed as quantitative differences in oxygen consumption. There is an increasing body of evidence, however, from studies of embryonic nutritional requirements (see below), that regional differences in visible developmental activity are correlated with both quantitative and qualitative differences in metabolism (including respiratory metabolism). Furthermore, Rulon (1935), Miller (1941), Moog (1943) and Spratt (1951a) have presented evidence for regional differences in respiratory activity by means of various indicator dyes. It has been shown by Spratt (1951d, 1951e) that the intensity and rate of reduction of neotetrazolium chloride per cell is greater in the node. It was found (1) by examination of whole blastoderms with reflected light and under an oil immersion lens; (2) by examining sections of blastoderms; and (3) by superposition of relatively inactive reducing regions, that the reduction patterns are not due simply to differences in number of cell layers or thickness of the regions (*cf.* Philips, 1942). It should be noted in this connection that a very similar pattern has been demonstrated (Spratt, 1950a), consisting of regional differences in sensitivity to anoxia (presumably the converse of differences in oxygen requirements). Regional differences in sensitivity to cyanide poisoning have also been described (Buchanan, 1926; Spratt, 1950b).

The failure of Philips to observe any differences in oxygen consumption in isolated regions of the blastoderm may be attributed to the effects of cutting. It is not inconceivable that dissection of the spatial pattern of developmental activity might dampen any underlying differences in metabolic activity by depressing activity in formerly active regions and stimulating it in formerly inactive regions. One is reminded in this connection of the effects of cutting on regeneration activities. In blastoderms dissected in the manner described by Philips (1942, FIGURE 2), the characteristic pattern of tetrazole reduction (Spratt, 1951a) disappears when the pieces are explanted *in vitro*, each isolate showing about the same reduction activity (Spratt, 1951f). Experiments are in progress to determine whether the combined reduction activity of the pieces is equivalent to that of a comparable whole blastoderm. It has also been observed that morphogenesis and cellular differentiation in such small isolates is greatly depressed (*cf.* Philips, 1942).

Carbohydrate Metabolism

Presumably, the early embryo can use simultaneously the reserve nutrients at its disposal, but evidence based on R.Q. measurements (Needham,

1932b), use of metabolic inhibitors (Needham, 1932c; Spratt, 1950b), studies of anaerobic glycolysis (Needham and Nowinski, 1937), and studies of nutritional requirements of explanted blastoderms (Spratt, 1948a, 1949a, 1950a), indicates that carbohydrate is the main source of potential energy during the first four days of incubation. However, from the studies of Needham (1932a) and Dickens and Greville (1933) it appears that this preference of the early embryo for carbohydrate is not due to any lack of capacity for protein breakdown.

In studies of the nutritional requirements of one to two-day-old blastoderms explanted *in vitro* (Spratt, 1948a), glucose was found to be the only nutrient present in a synthetic medium (containing also amino acids and vitamins) which was essential to continuation of the processes of morphogenesis and cellular differentiation (histogenesis) during 24-28 hours *in vitro*. In further studies (Spratt, 1949a, 1950b), it was shown that d-mannose, d-fructose, d-galactose, d-maltose, pyruvate, and lactate could replace glucose in supporting early development, but with decreasing effectiveness in the order given. Taylor and Schechtman (1949) have presented evidence for a non-dialyzable, non-enzymatic factor in yolk extract which can partially substitute for glucose.

A number of other carbohydrates and metabolites could not be utilized by the embryo for supporting its continued development, *e.g.*, sucrose, lactose, d-ribose; α -ketoglutaric, succinic, malic acids; fructose-diphosphate, glycogen, *etc.* (*cf.* Astrup *et al.*, 1947). Non-utilization of the phosphorylated hexoses and glycogen (*cf.* Needham and Nowinsky, 1937; Needham, 1942) apparently cannot be ascribed to any inability of the embryo to metabolize these compounds, in view of the capacity of extracts and homogenates of chick embryos to glycolyze hexose-di-phosphate (Meyerhof and Perdigon, 1940; Stumpf, 1947; Novikoff *et al.*, 1948). Indeed, the latter investigators have demonstrated the presence in three- to ten-day embryos of many of the phosphorylated intermediates of carbohydrate metabolism, as well as ATP, ADP, DPN, *etc.*

In recent studies (Spratt, 1951c), it has been shown that many substances, although not capable of supporting continued development of the explanted embryo, nevertheless can be metabolized to some extent (enzymatically dehydrogenated in the presence of methylene blue and neotetrazolium chloride) by uncubated and 24-hour blastoderms, *e.g.*, sodium salts of succinic, citric, malic, α -ketoglutaric acids; glucose-1-phosphate, hexose-di-phosphate; whereas no reduction of the dyes occurred when sucrose, lactose, sorbose, acetate, *etc.* were used as substrates.

Metabolic Basis of Developmental Processes

The chemical embryologist (but not necessarily the biochemist) thinks of embryonic development as consisting of a number of component processes: maintenance, growth, morphogenesis and cellular differentiation (histogenesis). These processes, although closely interlocked in normal development, are nevertheless separable, not only in thought but also experimentally. The question naturally arises as to whether there are quantitative and quali-

tative differences in the metabolic reactions underlying the different processes.

(A) *Differential Nutrient Requirements.* Until recently, little was known about metabolic changes underlying morphogenesis and cellular differentiation in the chick embryo. Some information is provided by experiments in which the nutritional environment of explanted embryos is altered or controlled (Spratt, 1947b, 1948a). In embryos explanted to carbohydrate media (glucose, Ringer, buffers) containing no nitrogen sources, there is little or no growth, yet morphogenesis (the form-building movements of cells) and cellular differentiation continue.

When the concentration of carbohydrate in the medium is lowered gradually, or when different, utilizable carbohydrates (glucose, mannose, fructose, galactose, pyruvate, *etc.*) are made available to the explanted blastoderm, a whole array of differential nutrient requirements for support of the component processes is revealed (Spratt, 1948b, 1950a). Furthermore, specific developmental events, such as formation of the heart, brain, spinal cord, optic vesicles, *etc.*, have both quantitatively and qualitatively different nutrient requirements. For example, the brain and spinal cord will not develop at concentrations of sugar substrate which are quite adequate, however, for supporting formation and pulsation of the heart. Also, the maximal degree of histogenesis in the brain and optic vesicles occurs only in the presence of an adequate amount of glucose. On the other hand, the development and pulsation of the heart is less exacting in its qualitative nutrient requirements. Here is an interesting situation in which nutritional differences characteristic of the adult heart and brain are present during the formation of these organs in the embryo.

As a group, morphogenetic processes (cell movements) require less exogenous, carbohydrate nutrient than processes of cellular differentiation (histogenesis). It has been suggested that there may be a fundamental, energetic difference between form-building and tissue-building activities in the embryo (Spratt, 1950b). It is fairly obvious that these differences in nutritional requirements reflect differences in metabolic mechanisms underlying the developmental processes and events.

(B) *Differential Effects of Inhibitors.* A further analysis of the biochemical reactions underlying the various developmental activities has been attempted by the use of metabolic inhibitors (Spratt, 1950b). Appropriate concentrations of iodoacetate, citrate, malonate, cyanide and azide prevent the formation or inhibit further development of the brain, but have little effect on heart-formation or pulsation. Fluoride has almost the opposite effect, namely, concentrations which cause degeneration of the heart have no appreciable effect upon the central nervous system. Presumably, these differences in the effects of inhibitors on different embryonic events indicate differences in the underlying metabolic pathways being used. In earlier studies on the effects of iodoacetate, fluoride, and cyanide on respiration, and glycolysis of the blastoderm (Needham, 1932c), no correlation with particular developmental processes or events was studied.

A further fact of interest and possible significance emerges from the nutri-

tional and inhibitor studies. Nutritional requirements for the maintenance of a differentiated structure (*e.g.*, brain, heart, *etc.*) are qualitatively (but not quantitatively) the same as those for its formation (morphogenesis and differentiation). This observation (Spratt, 1950a), together with the results of the inhibitor experiments in particular (Spratt, 1949b, 1950b), indicate that there is no qualitative difference between the metabolic basis (at least as regards the fundamental energy-yielding mechanisms) of the differentiation as compared with the maintenance of a structure. Theoretical implications of this concept of the "identity of maintenance and morphogenetic energy" have been discussed by Spiegelman (1945).

Spatial Patterns of Developmental and Metabolic (Enzymatic) Activity

As pointed out above, embryologists have long been aware of the spatial pattern of developmental activity characteristic of early chick (probably all vertebrate) embryos. This pattern consists mainly of two centers of morphogenetic and histogenetic activity, namely, the node (dorsal blastopore lip) and the anterior end of the neural axis (fore-brain). The node is the more active of the two in terms of visible cell movements and differentiations, because it is here that the major axial structures of the embryo are progressively laid down. We have here, at a single stage of development of the embryo as a whole, a topographic pattern of different stages in the development of particular structures and organs, a condition which would seem to be well-suited to analysis of the mechanisms of the developmental processes.

It seems reasonable to assume that this pattern of developmental activity is underlain by a corresponding pattern of physiological (metabolic) activity. Evidence for this is clearly found in the differential susceptibility of embryos to cyanide (Hyman, 1927) and differential staining with vital dyes (Child, 1925; Rulon, 1935; Miller, 1941). One of the criticisms often raised against the interpretation of these observations as indicating patterns of physiological or metabolic activity is based on the absence of any accurate characterization of the activity.

(A) *Response to Environmental Modifications.* Further demonstrations of the existence of metabolic activity patterns in the early blastoderm and, to some extent, a characterization of these are provided by experimentally modifying the environment of the explanted embryo. Such modifications all give rise to a characteristic pattern of differential degeneration and inhibition of development, whether they consist of: (a) variations in the kinds of substrates (nutrients) made available or substrate deprivation (Spratt, 1948b, 1949a, 1950a); (b) partial anaerobiosis (Spratt, 1948b, 1950a); (c) carbon dioxide deficiency (Spratt, 1949c); (d) lowering the pH of the medium (Spratt, 1950a); or (e) presence of metabolic inhibitors (Spratt, 1949b, 1950b). Moreover, there seems to be a significant coincidence between the time course and spatial pattern of degeneration and the time course and spatial pattern of developmental activity (Spratt, 1950b, FIGURE 2). Regions of the blastoderm where developmental activities are greater (*e.g.*, The

node and head-fold, or, later, the segmental plate and fore-brain, *etc.*), are more sensitive to adverse environmental conditions than are regions which have already passed through a period of developmental activity (*e.g.*, hind-brain, spinal cord, somites, *etc.*).

(B) *Localization of Reducing Enzyme Systems.* In studies in progress (Spratt, 1951f), an attempt is being made to characterize more specifically the pattern of physiological activity in terms of the distribution of: (1) amounts and kinds of chemical substances and (2) enzyme activities. In 20–30 hour embryos explanted to albumen or Ringer-glucose media containing the reducing enzyme indicators, triphenyl- or neotetrazolium chloride or potassium tellurite, the (aerobic) pattern of reduction of the colorless reagents to formazan (red or purple) and tellurium (black), respectively, coincides with the pattern of developmental activity. The anaerobic pattern of reduction of methylene blue is essentially the same. Thus, regions of embryos where developmental activities are greater (*e.g.*, the node and head-fold in head-process blastoderms; the node, segmental plate, posterior end of the primitive streak and fore-brain in early somite blastoderms; and the tail-bud in older embryos) have a more rapid and possibly greater capacity for reducing the enzyme indicators than other regions (Spratt, 1951a). There is some evidence from sub-developmental temperature studies (Spratt, 1951e) that these differential reduction patterns (differences in formazan distribution) are, in part, due to regional differences in rate of uptake of the tetrazole at optimal developmental temperatures and not to differences in passive penetration of the dye.

It is interesting that the node and fore-brain are also regions showing greatest cytoplasmic basophilia (ribonucleoprotein concentration?) when stained with toluidine blue (Gallera, 1948, Spratt, 1951e). In the studies of Moog (1943), the distribution of cytochrome oxidase activity (indophenol blue reaction) correlates well with the pattern of developmental activity. The node also is a region which stains differentially greater with Janus green, presumably a fairly specific indicator of mitochondria. When these observations, together with recent studies by Kun (1951) on the mechanism of enzymatic reduction of triphenyl tetrazolium chloride, are evaluated, they are suggestive of a possibly important association of dehydrogenase activity, the cytochrome system, and mitochondrial distribution with developmental activity.

When lower (non-toxic) concentrations of tetrazole are present in a nutritionally adequate medium, development of the embryo continues for about 20 hours. Under these conditions, not only the node and fore-brain show marked reducing activity (red color), but also structures or parts (somites, chorda, neural tube) which have developed out of the node area during exposure to the indicator, in contrast to other structures which had formed prior to exposure (Spratt, 1951a). This is exactly what one would expect from a knowledge of the method of primitive streak regression and its relation to the embryo body (Spratt, 1947a).

(C) *Differentiation in Reducing Enzyme Systems.* A still more specific characterization of the kinds of reducing enzyme systems and their localiza-

tion has been attempted (Spratt, 1951e) by incubating the explanted blastoderm in the presence of specific substrates (glucose, mannose, fructose, galactose, succinate, pyruvate, *etc.*) and neotetrazolium chloride. For example, differential patterns of reduction in 3-12 somite embryos are different from one another when different sugar substrates are available to the embryo. Thus, under aerobic conditions in the presence of glucose, mannose or pyruvate, the node, but not the brain and cord, is capable of reducing the tetrazole. On the other hand, both the node and the brain and cord show activity when galactose or fructose are available. Under anaerobic conditions, both the node and the brain and cord show activity with glucose, mannose or fructose but only the node shows activity with galactose. Indeed, only the node is capable of reducing the tetrazole in the presence of all the substrates used under either aerobic or anaerobic conditions. Here, apparently, is an interesting example of differentiation in reducing enzyme capacities between the node (a center of differentiation activity) and the brain and spinal cord (already partially differentiated structures). In other words, the node exhibits a wide array of specific reducing capacities, whereas the brain and cord presumably have lost some of these in the course of their development out of the node area.

It is interesting to point out, in concluding this review, that the developmental and enzymatic activity of the node may be correlated with its greater "differentiation potential" (used in the sense as defined by Spiegelman, 1945), greater array of tissue-forming potencies (Willier and Rawles, 1935; Rawles, 1936), and greater capacity for regulation and regeneration (Waddington, 1932; Spratt, 1940). As stated above, it is also the region most sensitive to unfavorable environmental conditions such as starvation. Even a fairly mild degree of starvation of the embryo (three to six hours' incubation in a non-nutrient Ringer solution) followed by explantation to a nutritionally adequate medium (containing tetrazole) results in a loss of all dehydrogenase or related enzyme activity in the node region, although other regions show typical reduction capacities (Spratt, 1951b).

Summary and Conclusions

(1) Advances in our knowledge and understanding of the metabolic processes underlying early embryonic development seem to depend upon a study of the biochemistry and metabolism of the embryo in relation to particular developmental processes, events or changes between stages.

(2) During the first week of development, there does not seem to be any significant change in over-all metabolic activity as measured in terms of oxygen consumption or reducing enzyme activity per unit nitrogen or per cell. Although the first week is a period of rapid organ and tissue development, changes in metabolic activities underlying particular developmental processes apparently are not necessarily reflected as changes in over-all metabolic activity.

(3) Visible, regional differences in developmental activity within the early blastoderm have not been demonstrated in terms of quantitative differences in oxygen consumption per cell, but there is an increasing body of evidence for the existence of regional differences in metabolism.

(4) Carbohydrate is the main source of potential energy during the first four days of incubation but not necessarily the only source. The embryo is capable of utilizing some carbohydrates, but not others, for support of its continued development *in vitro*. The order of activity among the utilizable substrates is: glucose = mannose > fructose > galactose = maltose > pyruvate = lactate.

(5) The component processes of development: growth, morphogenesis, cellular differentiation and maintenance, as well as particular embryonic events such as brain-formation, heart-formation, *etc.*, seem to be underlain by both quantitatively and qualitatively different metabolic processes.

(6) There is a great deal of evidence that spatial patterns of developmental activity characteristic of early chick blastoderms are underlain by corresponding patterns of physiological (metabolic) activity. It is possible to characterize the patterns of metabolic activity, to some extent, in terms of enzyme activities.

Metabolic activity in relation to form (structural organization) and function (developmental activity) has been the guiding theme in this review of the metabolism of the early chick embryo. It may well be the theme in a review of our knowledge of the metabolic processes characteristic of later stages of development.

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Discussion of the Paper

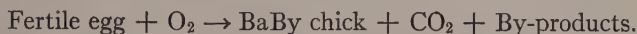
DOCTOR EDGAR ZWILLING, *University of Connecticut, Storrs, Conn.*: This may be an unfair question in some respects, but is there any relation between the inability of the small pieces of the blastoderm to differentiate and their loss of differentials in O_2 consumption and dye reduction? I was thinking in terms of retention of metabolic pattern and the ability of the tissue to differentiate. Does the tissue lose its ability to differentiate because it loses its pattern or are both losses independent? In the case of aggregates of hydroid tissue it seems evident that tissues will not differentiate unless a metabolic pattern is established.

METABOLIC PATTERNS IN EMBRYONIC DEVELOPMENT

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The description of the development of the chick embryo, in terms of the chemical events associated with its metabolism, lags far behind its description in morphological terms. The development, consisting of the conversion of oxygen from the atmosphere, plus the substances in the egg, into a baby chick, carbon dioxide and some other byproducts and residues, may be symbolized in a pseudochemical form as



It is the task of chemical embryology to unravel the numerous highly specific and detailed processes coupled into this reaction. We may suspect that most, if not all, of the reactions will be known from studies in other forms of living material and perhaps many details will be more readily studied in larger and, in some instances, less complicated forms of life. In the embryo, the chemical changes have a temporal sequence and the problem of development cannot be regarded as solved until a kinetic description of all of the changes between reactants and products is fitted into the twenty-one day period during which it occurs. This paper deals with some technics for the description of the intermediate phases which have seemed useful to us and which may have some general interest.

The process of development is spontaneous and occurs at a finite speed. Thermodynamics teaches us that such real and spontaneous reactions involve a loss of free energy and an increase of entropy in the system as a whole. It is no doubt true that the system bounded by the embryo itself (a better definition possibly would include all those materials in the egg which will actually be part of the embryo) shows an increased free energy and decreased entropy as a result of development. Both of these result from chemical degradation of other parts of the egg and of the atmosphere. This is a typical behavior pattern of living systems which characteristically utilize overall chemical degradation to produce some highly improbable arrangements of atoms to form the compounds of which they are constituted. However, thermodynamics is only concerned with initial and final states of a system. It is in the specific pathways of reaction that the peculiarities of embryonic development lie. To a large extent, these pathways are determined by the presence of catalysts and organizing elements. The enzymes may be regarded as the tools or machines operating upon the raw materials of the egg, using part as fuel to convert the remainder into the proper building materials and to combine them into the required structural pattern. A certain amount of the material is carried through reactions yielding free energy and the remainder raised to a higher energetic level. In this factory, we cannot distinguish easily between the fuel and the raw material for structure, again a situation typical of living systems.

It is possible to separate the partially completed product (the embryo) at

various stages of its development from the remainder of fuel, raw material, accumulated byproducts and "scaffolding" involved in the development. We can study the characteristics of the product as it varies with time and in chemical terms. When we follow the changes in reference to time, we are studying "growth."

Chemical growth, in a general way, may indicate the material increase within an organism of those things which are measured in chemical ways. A statement of the category measured is often useful so we can speak of weight growth, nitrogen growth, *etc.*

The literature of embryology contains many discussions on methods of describing the growth of embryos in time. Much of the discussion does not take advantage of certain elementary mathematical devices applicable to this problem. It should be obvious that in discussing continuing processes as complex as growth, the methods of calculus should be used and suitable scales devised. When we define growth as increase, growth rate becomes the growth produced per unit time. The weight growth rate is the increase of weight per day or per minute of development. It is characteristic of embryonic growth that it changes in rate with time. Almost always, therefore, a stated growth rate is only correct for a specific instant during development. The calculus provides a notation for this by writing the ratio of the infinitesimal growth in weight (for instance) as dw and dividing it by the infinitesimal time dt during which it occurred. The growth rate dw/dt can be a variable dependent upon time and other factors. Analytical geometry teaches us that dw/dt is the slope at a particular time of a plot showing weight as a function of time. One of the obvious variables upon which growth depends is the mass of growing material. Common sense tells us that there is a difference between a ten-pound baby gaining a pound in two weeks and an adult weighing 175 pounds achieving the same weight gain. The difference is not in the growth rate but in the multiplication rate, and we can formalize our common sense feeling by dividing the growth rate dw/dt by the weight w . It is the fraction of itself which the growing organism adds to itself per unit of time. However, $dw/w = d \ln w$. $d \ln w/dt$ becomes a constant related to the slope of a plot of $\log w$ against time. Thus semilog plots give us multiplication rates.

The aim of these considerations is to describe the growth of the organism in as simple a way as we can. In mathematical terms, it is desired to rectify the equation $w = f(t)$. Brody¹ has made much use of the semilog plot because he finds it possible to fit a series of straight lines to data so plotted. As long as the data sensibly follow such a line, the multiplication rate is sensibly constant. Abrupt changes occur which must be ascribed to the appearance of some new condition or to limitations imposed by the environment.

Another type of graph results when the logarithm of weight is plotted against the logarithm of time. This double log or log-log plot was used by Murray² to represent the wet weight of chick embryos. All of his data from five to 21 days of incubation fell on a straight line when so plotted. Of this plot, Murray wrote "when log weight is equated against log time, the points

approximated a straight line," but he did not "attach theoretical significance to the fact that the weights can be expressed by such a simple equation" namely $\log w = i + a \log t$.

It seems to me that Murray paid too little attention to the significance of this kind of plot and that we can derive some satisfaction from this simple equation. The slope on such plots is $d \ln w / d \ln t$, which we may call the accumulation coefficient.³ An example of this plot is shown in FIGURE 1.

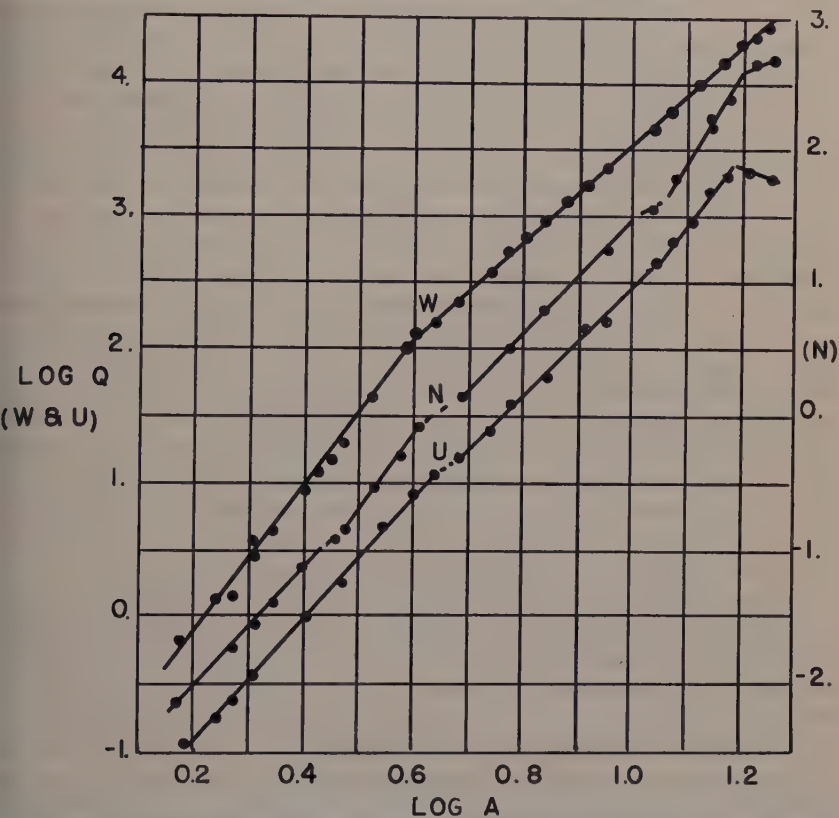


FIGURE 1. Accumulation diagram of weight, nitrogen and dipeptidase from (3). Log A refers to age of embryo in days. Log Q refers to quantity (W = weight, U = dipeptidase units). N = nitrogen. Log A = 0.64 at 4.33 days.

On this accumulation diagram, the wet weight data are accommodated by two straight lines, one valid from 1.5 to 4.33 days and the other from 4.33 to 21 days. There are thus two periods of constant accumulation coefficient for weight. These periods of constant accumulation are called phases. Interphases (4.33 days in wet weight data) are the times when an adjustment of the constants is required. It seems significant that several of the categories of chemical growth show nearly identical interphase times. Thus 4.3–4.5 days is an interphase found for the accumulation of wet weight, nitrogen and dipeptidase,³ aminopeptidase,⁴ cytochrome oxidase,⁵ and diphosphopyridine nucleotides.⁶ A figure intended to show the coincidence of in-

terphase times is given in FIGURE 2. If these coincidences can be considered real, each must correspond to a more or less drastic rearrangement of growth factors influencing the accumulation of not just a single category of growth, but of several or all of them.

An interesting possibility in the logical interpretation of the accumulation coefficient results when we remember that $dQ/Q = d \ln Q$ is the multiplication of the material Q . It is not complete nonsense to say that $d \ln Q/d \ln t$ is the multiplication rate of Q on a logarithmic time scale. We are accustomed to measuring time with a pendulum which is eminently satisfactory for the apparent movements of the sun and stars, trains, buses, airplanes, and the measurement of our working days; but it is commonly recognized that it is not completely satisfactory for physiological events. Some persons have proposed special physiological time scales.⁷ Let us see if there is any satisfaction in substituting a logarithmic for a linear scale in measuring time. As justification for the validity of such substitutions see the discussion of temperature scales in Lewis and Randall.⁸

We can agree that physiological time for any individual begins at conception. In the individual's physiological time, this is not properly called 0 but has the properties of $-\infty$ in that the individual did not exist before this point in time. (I choose to regard 0 as a number on a scale similar to any other number indicating an arbitrary point. The negative numbers are just as real in this sense as the positive ones and points designated by them are real points. The symbol $-\infty$ designates an imaginary point on a linear scale, a terminus which our number symbolism allows us to approach but not actually to reach.) As pendular time passes, he proceeds to his end by slower and slower steps. Both these "physiological" properties are characteristic of the behavior of a logarithmic times scale. Zero on the linear scale is $-\infty$ on the logarithmic one. Linear "one" is zero on the logarithmic. "Ten" linear is "one" on the logarithmic scale. We therefore propose the use of the logarithmic time scale as a physiological time scale which is independent of physiology but suitable for the measurements we wish to make.

The straight lines on log-log plots now mean that, in any given phase of accumulation, equal multiplication is accomplished in equal time (logarithmic units).

Needham⁹ has shown that data on the chemical growth of organisms can follow the heterogonic relationships of Huxley.¹⁰ That is, if y represents the amount of a particular chemical entity and x the amount of the "chemical totality" at the same time, then $\log y = \log b + k \log x$. Plots of x and y on double log scales give straight lines from which $\log b$ and k can be obtained. Needham doubted the physical significance of the relations on the basis of the dimensional characteristics of the equation but used them effectively in demonstrating certain similarities between the chemical growths of different animals. Lumer¹¹ adequately discussed Needham's doubts and showed that the equation is dimensionally valid. Waddington¹² considered the relationships of heterogonic functions to time functions and demonstrated that two entities whose multiplications are similar functions

of time will exhibit the heterogonic relation. Needham used the wet or dry weight on his log-log plots in the above-mentioned demonstration. These are similarly related to one another. Since the wet weight is a linear function of logarithmic time, so are all the other chemical entities considered by Needham. Thus, we may state the general principle. In a given environmental situation, the multiplication of a growing organism or any of its parts (either anatomical, as in Huxley's examples, or chemical, as in Needham's) is a linear function of logarithmic time. It is not difficult to imagine rather abrupt changes in the environmental situation of the chick embryo. The coming into action of the embryonic circulation, the exhaustion of a specific substance in the egg, the change of the functional kidney and, indeed, hatching are examples of such more or less abrupt changes of environment which would give rise to abrupt changes in multiplication rates and thus produce interphases.

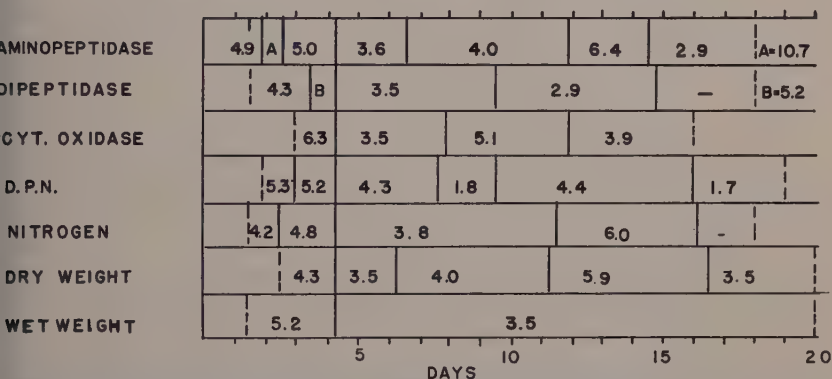


FIGURE 2. Each box shows the accumulation coefficient for the phase included. The terminations of the phases are indicated by the vertical lines. The extremes are at the limit of the data and are not necessarily termini of the phases.

If the notion of logarithmic time is found too bizarre, the accumulation coefficient can be rearranged to $d \ln Q/dt = k/t$. This states that the multiplication rate is inversely proportional to time measured from the beginning of development. It is as if the original germ-cell started with a certain fixed quantity of divisional (or multiplicative) potentiality which became more and more attenuated as time passed. The time required for each division is then prolonged in proportion to the time which has passed since conception, or, to the number of divisions which have occurred previously. The growth of an organism consists, however, of more than multiplication of the number of cells. The new cells increase in size as well. The increase must depend on the availability of nutrients and on the removal of wastes. In the chick embryo, the interphases may represent the times at which reorganizations of function and environment bring new factors into play so that new accumulation rates appear.

I suggest as a hypothesis that the metabolic patterns observed in the development of the chick embryo are best stated on a logarithmic time scale, that the patterns are dependent on several reorganizations in relationship

to the *intra ovo* environment and that, between such rearrangements, equal logarithmic time intervals account for equal amounts of multiplication. As support for this hypothesis, I cite the satisfactory fitting obtainable on log-log plots of growth data and the replication of the interphase time among the various quantities for which growth has been measured.

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THE DIFFERENTIATION OF ENZYMES IN RELATION TO THE FUNCTIONAL ACTIVITIES OF THE DEVELOPING EMBRYO

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Although the enzymology of the hen's egg and its embryo has been under investigation since the pioneer work of Herlitzka in 1907, the body of useful information what has been produced is still severely limited. We must discard many of the older observations because of the dubious character of the methods used. This is especially true where absence of an enzyme seemed to be demonstrated; but even where positive results were obtained, they were frequently expressed, not quantitatively, but merely by a plus sign, a symbol of somewhat restricted utility. Acceptable modern researches, moreover, often leave something to be desired in their fortuitous character. Many studies on embryonic enzymology have been exercises of an available technique rather than approaches to an embryological problem. We probably have learned more about the enzymology of the chick embryo, particularly in its early stages, from the studies of Spratt (1949, 1950), on the utilization of sugars, and Novikoff, Potter and LePage (1948), on phosphorylating glycolysis, than from direct investigations into the activity of specific enzymes.

If our knowledge of chick embryo enzymology is thus not sufficiently extensive to serve as a basis in itself for any comprehensive picture of embryonic metabolism, it is nevertheless sufficient to allow us to draw certain useful generalizations about the occurrence of enzymes and the way they accumulate. We can correct some persistent misconceptions about the vital processes of the embryo through these generalizations and replace them with a better understanding of the relation of enzyme changes to embryonic physiology.

1. *Young Embryonic Tissue Is Enzymatically Complex.* Although the old notion that the early embryo is virtually an enzymatic blank has long since disappeared, the view that very young tissue contains only a few enzymes, and these of low activity, still seems to pervade the thinking of many chemical embryologists. Yet, a variety of enzymes has been demonstrated, even in the first-day blastoderm, as TABLE 1 shows, and more have been found by the middle of the second day. Where so many enzymes of different functional significance can be demonstrated, it is certainly reasonable to assume that others are also present. It is probably significant that no enzyme has yet been shown by sufficiently refined methods to be absent during the first day of incubation.

2. *Enzymogenesis Proceeds at Differential Rates.* Not only are many enzymes present in early development, but they are also often more active, at least potentially, in early stages than in late ones. Although this fact is frequently obscured by the way the data are presented, it has been convincingly demonstrated by Levy and Palmer (1940, 1943), whose studies

reveal that, during the first few days of development, the embryonic tissue becomes poorer, not richer, in dipeptidase and aminopeptidase. It can be shown that this situation is not exceptional by treating the data on other enzymes, for which the time course of accumulation* has been described, in such a way as to bring out changes in accumulation rates. To do this in comparable fashion for different enzymes, I have calculated the activities of various enzymes in terms of numbers of appropriate units per mg. nitrogen, and then have taken the highest activity during the first week of incubation as 100 per cent. The results of this analysis of the available data are given in FIGURES 1, 2, and 3.

These three figures sum up several important facts about the nature of enzyme accumulation in early development. First, each enzyme accumulates at its own rate, which does not parallel that even of other members of the same group. Compare, for example, cytochrome oxidase and succinox-

TABLE 1
ENZYMES IN THE EARLY CHICK EMBRYO

<i>Enzymes demonstrated during the first day of incubation</i>	
Amylase	Galwialo 1926
Lipase	
Protease	
Cytochrome oxidase (Nadi reaction)	Moog 1943
Alkaline phosphatase (Gomori reaction)	Moog 1944
Acid phosphatase (Gomori reaction)	Moog 1944
<i>Enzymes demonstrated during the second day of incubation</i>	
Cytochrome oxidase (manometric)	Albaum, Novikoff and Ogur 1946
Dipeptidase	Levy and Palmer 1940
Aminopeptidase	Levy and Palmer 1943

dase, dipeptidase and aminopeptidase, alkaline and acid phosphatase. Secondly, continued development is not simply a matter of steady enrichment of enzymatic content, as has already been pointed out. After three days, many enzymes seem to increase in relative concentration, but, before that period, decrease in concentration is a somewhat general tendency. In most cases investigated so far the overall activity in later development does not rise above its maximum in the first week. Thirdly, there are few long-continued trends, most enzymes passing through a series of phases of elevation and decline instead. Only arginase follows an unchanging course, perhaps because it is associated with cell proliferation, as Edlbacher (1938) has suggested.

All the results embodied in FIGURES 1 to 3 were obtained with homogenates or extracts in which the normal structure of the tissue is destroyed. This method, if adequate quantities of substrates and cofactors are supplied,

* Enzymes are generally measured by study of the course of the enzyme-controlled reaction in homogenates or extracts of tissue. Under such conditions it is not certain whether a change in activity means that the enzyme has changes in molar concentration, or that it has altered in turnover rate. In rapidly growing embryonic tissues it is certainly reasonable to assume that new enzyme molecules are being synthesized. Nevertheless, the possibility remains to be tested that observed changes in enzyme activity with development are due in part to changes in the characteristics of enzyme molecules already present.

presumably measures the highest potential activity of each enzyme, but does not necessarily reflect the true activity *in vivo*. How great the disparity may be is suggested by the work of Spiegelman and Steinbach (1945), showing that breis of newly fertilized frogs' eggs consume oxygen ten times faster than do the intact eggs. That structure should have an equally severe restraining* effect in chick embryos, however, seems less likely, because the chick is not faced with the problem of maintaining order in a system in which

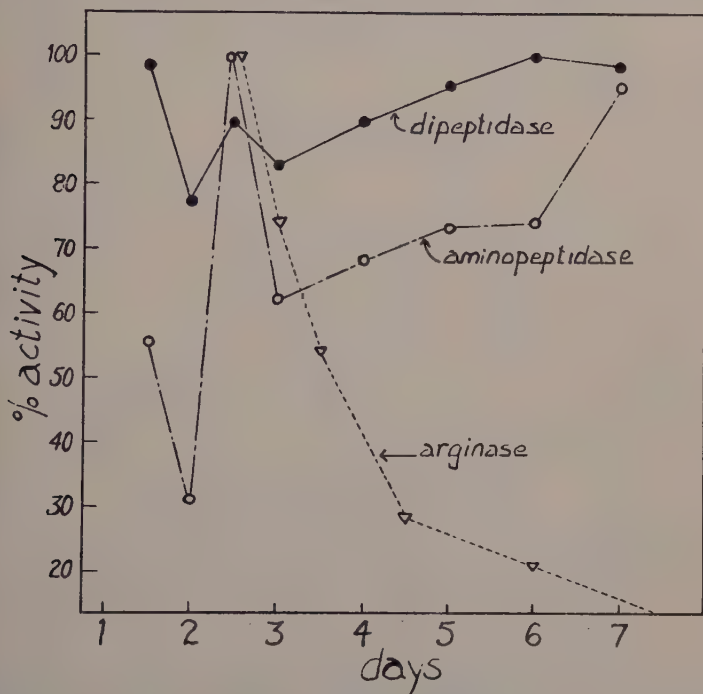


FIGURE 1. Enzymes associated with protein metabolism. In the preparation of this and the two following figures, enzyme activities were calculated in appropriate units per mg. N, and the highest activity during the first week was taken as 100 per cent. In the case of these enzymes, the activity during the remainder of the incubation period did not rise above the maximum value during the first week. Cathepsin is also present between four and seven days but its activity is extremely low (Mystkowski, 1936). Data on peptidases from Levy and Palmer (1940, 1943); on arginase from Brachet and Needham (1935).

cytoplasm and yolk are mixed together. Actually, the intact chick embryo seems to consume oxygen faster, per mg. nitrogen, than does its brei in the cytochrome oxidase assay. This fact is brought out in TABLE 2, which also reveals that the previously demonstrated decline in cytochrome oxidase activity from one to three days is paralleled by decline in rate of oxygen uptake by the intact system. Presumably the relatively low value of the whole 2-hour embryo is due to the inadequacy of diffusion in such a large mass of tissue. That the rate of oxygen consumption of the intact embryo falls during development is also revealed by the studies of Needham (1932) on

* Certain mature tissues, particularly those which are not continuously active, may contain enzyme reserves that are mobilized in times of stress. Chick embryonic tissues, however, live under standardized conditions and so may not be provided with reserves to meet emergency conditions.

the respiration of the whole egg. Although this kind of comparison between *in vivo* and *in vitro* activity cannot be made now for any other enzyme, this one example permits us to place some confidence in the physiological significance of the enzyme patterns pictured in FIGURES 1 to 3.

At the present time, how far can we identify the physiological significance of the enzyme patterns thus illustrated? For the first three days of development, the possibilities are not now very great. This period, in which several enzymes decline in relative concentration, is also characterized by a

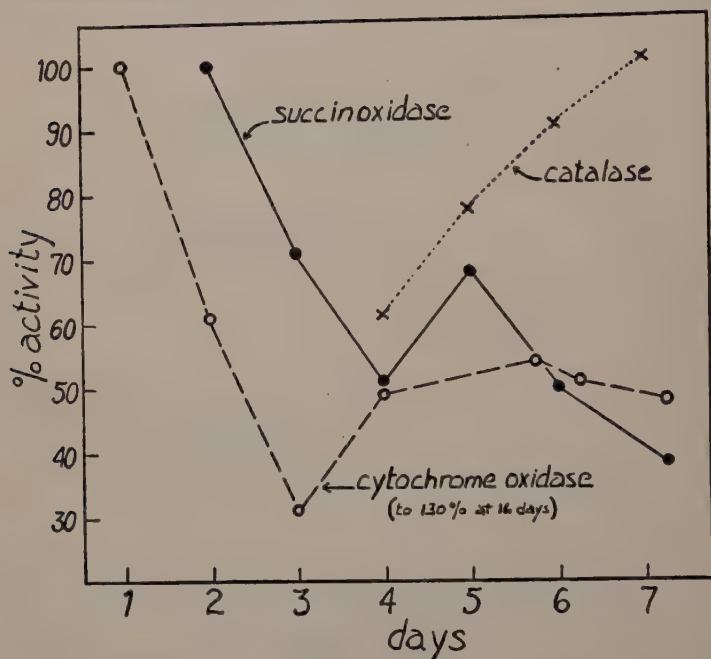


FIGURE 2. Oxidizing enzymes. At 16 days, cytochrome oxidase rises to a maximum value which is higher than its first week maximum. In calculating activities for catalase, succinoxidase, and cytochrome oxidase from one to three days, it was necessary to supply figures for weight and nitrogen concentration, since the authors did not give this information. Data on catalase from Kleinzeller and Werner (1938); on succinoxidase and cytochrome oxidase from one to three days from Albaum, Novikoff and Ogur (1936); on cytochrome oxidase from three to seven days from Levy and Young (1948).

great increase in water concentration, a fact which suggests radical changes in the protein structure of the cells. It is also the period of the most fundamental differentiations, because in this time the embryo is converted from a set of germ layers into a visibly differentiated body with all major parts represented. What the interrelations of these various facts may be, however, remains for the future to establish.

The meaning of the enzyme patterns of later stages, however, is easier to understand, at least in part. With continued differentiation, each enzyme tends to concentrate preferentially in some tissues or organs. Even at two days, tissues differ markedly in their alkaline and acid glycerophosphatase activity, as revealed by the Gomori technique (Moog, 1944) and, at three

days, the brain epithelium is substantially richer in dipeptidase than is the surrounding mesenchyme (Palmer and Levy, 1940). These facts and others of the same sort make the measurement of enzyme activities of embryo as

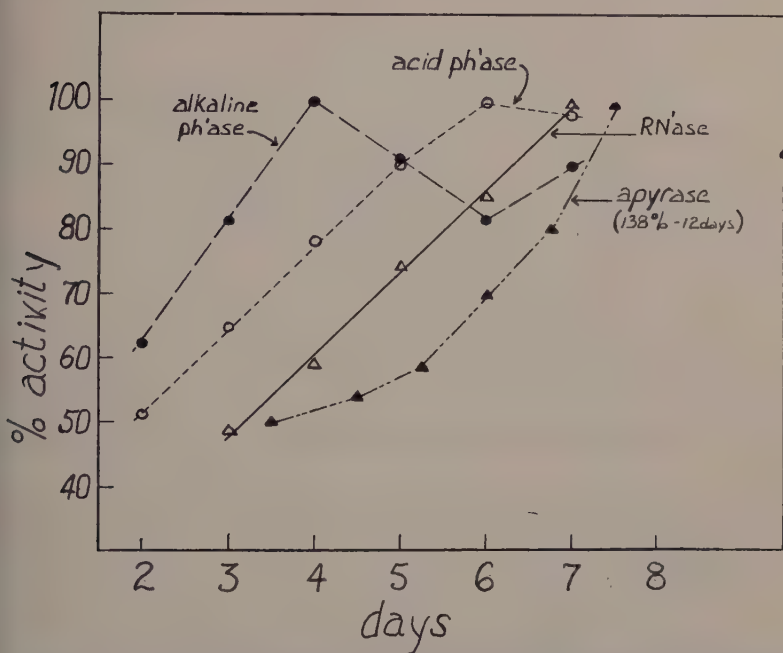


FIGURE 3. Alkaline and acid phosphatases (Moog, 1946), adenylypyrophosphatase (Moog and Steinbach, 1945), and ribonucleinase (Bernstein, 1950). Only apyrase is known to rise later to a value above its first-week maximum.

TABLE 2
RATE OF OXYGEN UPTAKE IN EARLY STAGES

Age in hours	cmm O ₂ /mg N/hr.	
	Intact embryo (with glucose)	Homogenate (with ascorbic acid)
25	1230	550
48	808 (-39%)*	305 (-40%)
72	230 (-82%)	159 (-68%)

* Figures in parentheses indicate the percentage decrease from the preceding day. Values for intact embryos are calculated from the data of Philips (1941), those for homogenates from Albaun, Novikoff and Gur (1946).

whole (a method that has been aptly called the "actuarial" approach to chemical embryology) steadily less meaningful as development proceeds.

3. *Enzyme Accumulation Is an Aspect of Functional Differentiation.* A study of the localizations of alkaline phosphatase within the body (Moog, 1944) may be used to illustrate the striking non-uniformity of the distribu-

tion of an enzyme even in early stages. Up to the middle of the second day all ectodermal and mesodermal materials are moderately rich in phosphatase, with the endoderm somewhat less so. The myocardium, however, is phosphatase-negative as soon as it differentiates, and the collecting ducts of the mesonephros, the liver, and skeletal musculature also become negative by the end of the first week. During the same period, however, the endothelium, the brush borders of kidney tubules, the sites of bone formation, and other loci become far richer in the enzyme than any undifferentiated tissue. Still other sites, however, as FIGURE 4 shows, undergo no substantial alteration in their phosphatase activity. Acid phosphatase shows a similar pattern of changing distribution with continuing development.

The invariable presence of the phosphomonoesterases in undifferentiated tissue implies that these enzymes are involved in some way in a general chemical mechanism underlying the visible alterations we recognize as differentiations. This conclusion is reinforced by the fact that the enzymes are present in very low concentration if at all, in many fully differentiated tissues. Apparently, these enzymes are not important in a maintenance metabolism distinct from an activity metabolism of some sort. Differentiation, of course, can be regarded as the proper functional activity of young embryonic tissue.

The key to the increases and decreases in phosphatase activity indicated in FIGURE 4 is found similarly in the involvement in the functional activity of the differentiated organs. In sites where the enzyme is known to be concentrated in mature tissues, it becomes concentrated just as function begins. Conversely, in tissues from which the enzyme is absent, or almost so, in mature stages, it disappears when functional differentiation occurs. Also, where tissue remains in a primitive state of differentiation for a long time, the level of phosphatase activity persists unchanged. The mesonephros provides a series of examples of these situations. During the first half of the fifth day, when tubular secretion is just beginning (Gersh, 1937), the enzyme becomes highly concentrated in the newly differentiated brush borders and, as suddenly, falls to a very low level in the cells of the tubule walls. The undifferentiated nephrogenous tissue, meanwhile, retains the moderate level of phosphatase activity characteristic of the whole organ at earlier stages.

To what extent are changes in activity of other enzymes during the late embryonic and fetal periods related to functional differentiation? If preparation for function dictates when an enzyme shall increase, and how fast, then we can make three predictions about the pattern of enzyme accumulation in organ development. First, the same enzyme will be expected to have different patterns of increase in different organs; second, within a single organ, several function-associated enzymes will be expected to have similar patterns; and, third, where the functional history of an organ is understood or can be readily inferred, enzyme accumulation will be expected to show a clear-cut relation to the functional activity.

The number of data by which these predictions can be tested is at present extremely limited. The results presented in FIGURE 5, however, show at

least that the first prediction above is accurate and that the others are not without support. In the preparation of the figure, the enzyme activities,

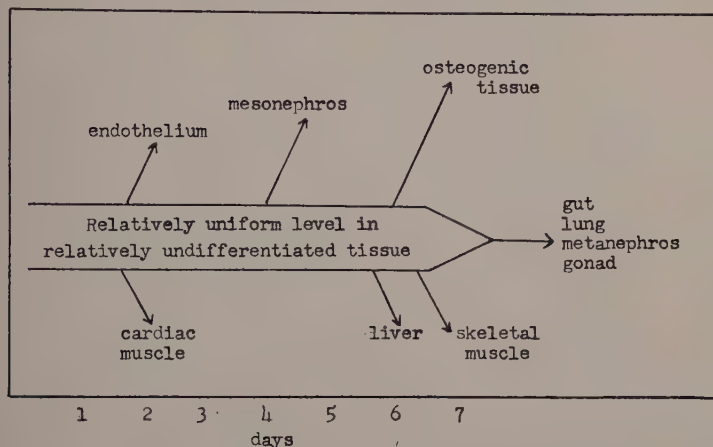


FIGURE 4. Diagram to illustrate shifts in alkaline phosphatase distribution during the first week of incubation. The enzyme is moderately active in all undifferentiated tissues, but increases or disappears when functional differentiation occurs.

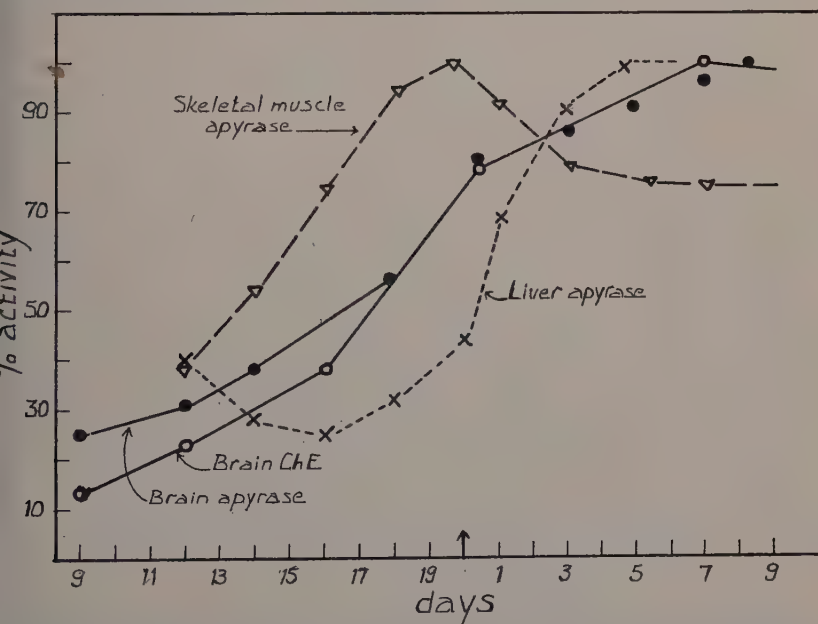


FIGURE 5. Adenylpyrophosphatase in brain, liver, and skeletal muscle, and cholinesterase in brain. The arrow indicates hatching. Here 100 per cent represent the highest determined activity, with all activities being calculated per mg N. Apyrase data from Moog (1947); cholinesterase data from Nachmansohn (1939).

again calculated as units per mg. nitrogen, are expressed in percentages with highest demonstrated activity taken as 100.

The figure clearly illustrates that the pattern of differentiation of adenyl-

pyrophosphatase (apyrase), the ATP-splitting enzyme, is peculiar to each of the three organs in which it has been studied. Recently, carbonic anhydrase also has been shown to develop at different rates in different organs (Clark, 1951). For the brain, FIGURE 5 reveals also a striking similarity in the accumulation of the two enzymes that have been closely studied in that organ. Cholinesterase is, of course, an enzyme of undoubted functional significance in nervous tissue. What the role of apyrase is in the brain is less certain, but it is suggestive that ATP serves as energy-donor in the acetylation of choline (Nachmansohn and Machado, 1943). Thus, the data seem to indicate that systems for synthesizing acetylcholine and for splitting it are built up in parallel.

Correlations between enzyme differentiation and organ function may also be drawn from FIGURE 5. It is particularly clear that, in skeletal muscle, where apyrase (ATPase) is known to be an important functional component, the enzyme reaches its maximum level on the very day that the muscles are first called upon to work in the business of hatching. In the brain, on the other hand, no equally sharp maximum is to be found in either functional activity or enzyme accumulation. A gradual functional maturation, rather, is suggested by the increased alertness and activity that the young chick shows during its first week out of the shell. Within the spinal cord, cholinesterase undergoes a five-fold increase in the period, between five and ten days, when reflex activity and spontaneous muscular movements first appear (Wenger, 1951). Wenger's work is particularly interesting in that it was done by a micromethod that can be applied to tissue samples containing no more than one microgram of protein.

The multiplicity of liver functions makes the results for this organ more difficult to interpret. Further, the rise in apyrase activity just after hatching occurs only in the presence of calcium, the enzyme (or system of enzymes) becoming sensitive to activation by calcium ions at 20 days. The increased activity, therefore, may be due either to a change in the characteristics of an enzyme already present, or to the synthesis of a new calcium-sensitive enzyme. In either case, the alteration is probably associated with a shift in liver functions which hatching, with its attendant nutritive changes brings about. That hatching is a critical period for the liver is also suggested by the fact that xanthine oxidase, previously absent, rises to a very high level of activity during the period of about 12 hours while the chick is making its way out of the shell (Morgan, 1930). Of course, the liver has assumed some of its functions much earlier, perhaps even at the beginning of the second week (Dalton, 1937); at that time, peptidase activity rises suddenly (Dumm and Levy, 1949), and the histochemical pictures indicate that acid phosphatase does so too (Moog, 1944).

One more case which illustrates the way in which enzyme accumulation is involved in functional differentiation is that of the alkaline phosphatase of the small intestine (Moog, 1950). The intestinal mucosa has long been known to be the richest source of this enzyme, where it appears to be tremendously concentrated in the striated borders. Not until the middle of the third week, however, does the enzyme begin to rise above the primitive

level (FIGURE 4). Then it suddenly sweeps upward, increasing almost 75-fold (calculated per unit nitrogen) within 60 hours, so that just after hatching the level of phosphatase activity is higher than that found in the adult. Thus, the newly-hatched chick sends the first food it eats into an intestine that is fully prepared, at least as far as phosphatase content and localization are concerned.

From these various cases, we can begin to derive some specific understanding of the meaning of patterns of enzyme accumulation in whole embryos and organs. In those instances in which we know something about the functioning of an organ in developmental stages, and know also that a given enzyme is involved in the function of that organ in its mature state, we can see that the differentiation of the enzyme is directly related to the differentiation of function. No case has yet been found in which such parallelism does not appear where it is to be expected. Of course, this conclusion is interesting to the study of enzymogenesis itself; but it may also be important as a guiding principle in the study of fetal physiology. At the present time, our understanding of the functional status and activity of the organs of the chick embryo from stage to stage is very limited. Enzyme patterns may suggest where critical changes are occurring in the vital activities of developing organs and tissues and, by pursuing such leads, we may be able to increase considerably our knowledge of the physiology of the fetus. In even more general terms, since the developing organ reveals the transition from non-functional to functional states, such studies may also contribute to our understanding of the relation between enzyme action and tissue function in adult life.

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APPEARANCE OF TISSUE-SPECIFIC PROTEINS DURING DEVELOPMENT

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Introduction

Chargaff (1945) has described the development of a scientific problem as passing through four stages: (1) formulation; (2) oversimplification; (3) anarchy: a period of chaos with little tendency for convergence of opinion; and (4) solution. Almost invariably, solution or partial solution of a given problem leads to a modification and restatement of the original concept and hence to a repetition of the cycle. The evolution of our present state of knowledge in the field of morphogenesis is an excellent case in point. Following the classical studies of Spemann, inductive effects exerted by one embryonic tissue, on an adjacent tissue less advanced along its course of differentiation, have been extensively explored. The results of nearly thirty years of observation and experimentation lead to the conclusion, emphasized by McKeehan in a recent publication (1951), that the inductive effect depends upon a chain of interactions between tissues, rather than upon a single event which would effect "induction" as a single response. For the majority of students in the field of morphogenesis, the phase of oversimplification, characterized by the search for single chemical mediators of induction, is past. The present period is marked by a lack of unanimity of opinion as to either the nature of the interactions which characterize the inductive process or the activating mechanism which sets off the chain reaction in the course of normal development. However, recent contributions from several disciplines within the broad framework of developmental biology have prompted a convergence of opinion concerning one facet of the problem, viz., the high degree of selectivity in the inductive process. It has become increasingly evident that molecular configuration is intimately involved in biologic specificity (Haurowitz, 1949; Irwin, 1949; Weiss, 1950). There can be little doubt that one of the central problems of developmental biology is the determination of the structure and properties of protein and conjugated protein molecules. Inextricably interwoven with the problem of the structure of proteins is the process by which they are synthesized in cells. These remarks are directed toward the solution of some of the questions raised by an analysis of the time and manner of origin of tissue-specific proteins, and an analysis of the role of protein molecular rearrangements in the growth and differentiation of the chick embryo.

Some of the pertinent questions follow: What proportion of the vast array of specific proteins of the adult organism is represented in the egg and embryo prior to the formation of organ primordia? Does the failure to demonstrate the presence of some complex molecules in the early embryo necessarily indicate their absence, or merely that they are present in bound form,

* Contribution Number 483 from the Zoological Laboratories, Indiana University.

from which they are subsequently dissociated during ontogeny? Are any proteins which disappear in later stages found in the embryo? What are the relative roles played by the globular and fibrous proteins in embryonic determination?

It is clear that development is characterized by progressive change in the complement of the proteins and other complex molecules of the embryo. Experimental evidence in support of this concept is derived from several sources. There are incisive examples of progressive change in the activity of well-defined enzyme systems during embryonic development (Boell, 1948; Flexner and Flexner, 1948; Moog, 1947). Certain enzymes are either absent or present in minute quantities in the egg and early embryo and only become clearly demonstrable later in development. The blood proteins also have been extensively studied in this regard. The serum protein fractions of amphibians, birds and mammals change considerably during ontogeny (Cooper, 1946, 1948, 1950; Nace and Schechtman, 1948; Moore, Shen, and Alexander, 1945). The extent to which the observed changes reflect synthetic processes, and not the separation of complex molecular associations, has not been established.

A similar situation prevails with respect to the development of tissue-specific proteins, with the further disadvantage that fewer unequivocal instances of change in number, kind, or distribution of tissue-specific proteins or conjugated proteins during ontogeny have been described. Certain tissue-specific antigens are present in the embryo well in advance of the appearance of the corresponding organ primordia; yet not all molecular species of the adult can be detected in early embryonic stages, or, in fact at any time during embryonic life. The limited data available point to the tentative conclusion that quantitative and qualitative differences in tissue-specific antigens occur during ontogeny. In this case, ontogeny is used in the broad sense, including post-embryonic development. Before examining the experimental evidence which supports this conclusion, we shall direct our attention briefly to the techniques which have been employed in the analysis of the ontogeny of tissue-specific antigens.

Among the most precise techniques available for the study of biologic specificity, as related to molecular composition, are those from the field of immunochemistry which enable the identification of well-defined tissue components, *e.g.*, proteins and conjugated proteins (proteins bearing smaller molecules as haptens or combining groups). The techniques involved frequently require analysis of isolated systems. Therefore, in order to relate the properties of a given component to the dynamic state of the tissue from which it is derived, it is necessary to provide supplementation by the use of other techniques. Whenever possible, physiological criteria of differentiation should be emphasized, because morphological criteria frequently appear too late to reveal basic differences among similar cellular components. In addition to the necessity for the application of as many relevant tools as can be brought to bear on the problem, other precautions must be strictly observed. Although the excellent demonstration by Waugh (1948) of the reversible modification of insulin by relatively drastic treatment ordinarily

associated with irreversible change in the properties of proteins indicates that some revision of our traditional concept of denaturation may be in order, the fact remains that many proteins are so unstable that they are susceptible to alteration by seemingly minor environmental changes. Therefore, the extraction and purification of tissue proteins must be carried out at low temperatures, employing suitable mild solvents. Moreover, since tissue components are rarely obtained in crystalline form, careful attention must be paid to proper absorption of antisera in order to insure their specificity.

The discussion which follows is concerned mainly with observations on the development of the chick, although it is realized that other equally pertinent illustrations of many of the phenomena considered have been described. No attempt is made to provide a complete survey of the literature, since these remarks are not intended to constitute a review of past achievements, but are rather a progress report, pointing out the gaps in knowledge in the field and indicating promising lines for future study. The discussion includes numerous unpublished observations, with observations previously published only in abstract form.

Tissue-specific Antigens in the Early Chick Embryo

Immunological Studies. The first clear-cut observation of the presence of organ antigen in the early chick embryo was made by Schechtman (1948), who reported that substances of common antigenic character, occurring in saline extracts of brain, heart, liver, and muscle of the nearly-hatched (19-20 days) chick, are also present in extracts of primitive streak and neurula stages of the chick embryo. This common organ antigen, which is saline soluble, was shown to differ from the antigens of both blood and yolk, and from the Forssman antigen. Evidence derived from a study of absorption and precipitin tests employing anti-chick brain sera in combination with homologous or heterologous tissue antigens or with extracts of early chick embryos indicates that the antigen is not organ-specific in the sense of being limited to brain alone, but is best described as a general organ antigen. Of six anti-brain sera, four showed no activity with the homologous antigen after absorption with heterologous organ extracts (heart, muscle). Only one anti-brain serum showed brain-specificity after absorption. Extracts of the early chick embryo reacted positively with anti-brain sera absorbed with whole blood. Absorption with extracts of brain, heart, or liver, however, removes all precipitin for embryonic tissues, indicating that the early embryo contains a general organ antigen.

These results have been confirmed and extended (Ebert, 1950). From a consideration of the foregoing data, it appeared likely that the organ antigen of the early embryo described by Schechtman (who used only anti-brain sera which exhibited little or no tissue-specificity) might be more accurately characterized by employing antisera of more clearly defined specificity. Three types of anti-organ sera, therefore, namely, anti-heart, anti-spleen, and anti-brain sera, variously absorbed, were utilized in combination with extracts of the early chick blastoderm. Despite the fact that each antiserum was highly class-specific and quantitatively organ-specific (Ebert, 1951a), the objective

of the investigation was not immediately achieved, because the results of immunological (precipitin) tests differed very little from those previously described. Absorption of any of the three types of antisera with extracts of heart, brain, or spleen removes precipitins for extracts of embryonic tissue (definitive primitive streak and early somite stages), despite the fact that absorption with heterologous antigen does not remove precipitins for the homologous antigen (Ebert, 1950, p. 356). It appeared, therefore, that the organ antigen of the early blastoderm was of a more general nature than any of the tissue-specific antigens described. In only one respect did the results differ from those reported by Schechtman. Absorption of antisera with extracts of adult liver did not remove precipitins for either the adult tissue-specific antigens or the embryonic antigen. Schechtman observed that anti-brain sera absorbed with liver extracts showed a complete loss of precipitins for embryonic antigens. The most probable explanation for the discrepancy appears to be the difference in method of preparation of antigens for injection and test purposes (freshly extracted *vs.* lyophilized antigens, respectively).

*The Effects of Anti-organ Sera on the Development,
in vitro, of the Early Chick Blastoderm*

The failure of the immunological analysis to reveal specific differences in the organ antigens of the early embryo led to the adoption of a second approach which has been utilized effectively in determining the time of appearance of tissue-specific antigens in the early embryo. These efforts trace their origin to the need for extension of the suggestive results obtained by Weiss (1939, 1947) in experiments aimed at testing the template concept of growth (a generalized hypothesis of growth catalysis by complementary template systems, comparable to antigen-antibody relations). When small doses of antisera, produced against autolysed extracts of adult chicken organs, were injected into developing chick embryos, Weiss observed a significant weight increase in the homologous organ, despite a general depression of growth. In view of the established tissue-specificity of the three types of antisera described above, and from a consideration of the specific effects of antisera on the intact embryo reported by Weiss, it appeared likely that exposure of the early embryo to specific anti-organ sera might prove to be a more sensitive method for detecting specific antigens than the immunological procedures previously outlined. The primary objective of the research was to determine whether specific inhibitory effects might be produced on definite areas of the early blastoderm cultivated in the anti-organ sera. Basic information with respect to the presence and distribution of specific antigens is necessary as a background for further studies concerned with possible growth-stimulatory effects of antisera.

To date, over 600 blastoderms (definitive primitive streak to early somite stages) have been explanted to media (Spratt, 1947, 1948) containing normal (control) or anti-organ sera. A wide range of serum dilutions has been tested. The principal results of the investigation may be briefly summarized as follows: Definitive primitive streak and head process stage embryos

explanted on media containing any of the three types of anti-organ sera in high concentration rapidly undergo disorganization characterized by (1) the loss of gross structural features and (2) cell clumping or agglutination. There is no indication of any "pattern" of disorganization, nor has a "primary center" from which the effect spreads been observed. While head fold and 1-6 somite stages are similarly affected, there is evidence of an anterior-posterior gradient of susceptibility.

At critical intermediate concentrations of antisera, relatively specific effects on the blastoderm are observed. The effects of anti-heart and anti-spleen sera differ sharply from the effects resulting from treatment with anti-brain sera. The former affect primarily mesodermal elements of the blastoderm resulting in embryos lacking in somites and, to a considerable extent, lateral plate mesoderm. The nervous system is "intact" but its degree of organization appears to be directly related to the extent of mesodermal inhibition, *i.e.*, the earlier the stage explanted, the more marked is the inhibition of mesodermal structures, and the more abnormal is the nervous system. In contrast, anti-brain sera affect chiefly nervous tissues.

The most specific effect observed to date is best illustrated by a comparison of the effects of anti-heart and anti-spleen sera. Although both antisera affect mesodermal tissues in general, blastoderms explanted to media containing anti-spleen sera in critical intermediate concentrations develop a pulsating heart, while the heart is absent or extremely retarded in blastoderms explanted to anti-heart sera.

The only effect of any of the antisera at relatively low concentrations is a generalized inhibition of growth as determined by comparison of camera-lucida drawings. Frequently, the inhibition is so marked that dwarf embryos, no larger than the primitive streak stage explanted, but otherwise normal, are obtained.

It must be emphasized that the nature of the inhibitory effect produced is dependent on the final concentration of antiserum employed. There is no reason to believe that those antibodies produced against specific tissue antigens are not active in high concentrations, but rather that their specific effects are masked by the more generalized inhibition of the blastoderm. Specific effects at intermediate concentrations, then, are due to the fact that the non-specific antibodies are diluted out of their active range, while specific antibodies retain their activity at greater dilutions. It is well known that injection of a complex mixture of tissue antigens results in the production of antisera containing a multiplicity of antibodies. Moreover, different antibodies may be present in different relative concentrations. It is not surprising, therefore, to find considerable variability and overlap in the activity of different groups of antisera tested. The following scale of serum concentrations, however, may serve as a guide: (1) Non-specific lethal effects: anti-heart and anti-spleen sera in final concentrations of from 1:15 to 1:100; anti-brain sera, 1:15 to 1:50; (2) specific effects: anti-heart and anti-spleen sera, 1:80-1:110; anti-brain sera 1:30-1:65; (3) non-specific growth-inhibition: anti-heart and anti-spleen sera 1:80-1:200; anti-brain sera 1:30-1:100.

All concentrations of anti-heart and anti-spleen sera between 1:200 and

1:600 allow essentially normal development. Similarly, concentrations of anti-brain sera above 1:100 are ineffective (tests to date range up to 1:480). The analysis has thus far produced no evidence of stimulation by low doses of antisera. Since this failure may be due to a lack of proper criteria of stimulation in the systems analyzed, experiments toward this end are being continued with other antigens.

Immunochemical Studies Employing Highly-purified Proteins

It follows from the foregoing that a major limiting factor in the production of specific inhibitory effects of antisera is the degree of purity of the antigens employed. The question next arises, therefore, as to the selection of highly purified antigens for analysis. Only one of the three groups of tissue antigens already described, the heart-specific group, has been selected for further study. The immediate objective of the research is to determine the relationship of the heart-specific antigens to the proteins recognized as being concerned in muscle contraction, actin and myosin. The initial approach has been directed toward the preparation and standardization of antisera against highly purified actin and myosin of chick cardiac muscle. As sources of the purified proteins, 18-day embryonic chick and adult chicken heart have been used. The extraction procedures followed are those described by Straub (1943) and Mommaerts and Parrish (1951). Despite the fact that Kesztyüs, Nikodemusz, and Szilagyi (1949) have reported that both actin are antigenic, the rabbit antisera produced against purified actin and myosin in our laboratory have been so impotent as to be of no value. In fact, titers significantly higher than the range of natural tissue antibodies have not been obtained. In view of the positive report of Kesztyüs *et al.*, a more extensive series of experiments is now in progress in an effort to account for these negative findings.

It will be of interest to consider next a conjugated protein which, on the basis of antigenic specificity as revealed by immunochemical analysis, shows promise of fulfilling the need for a specific antigen of known embryological origin and fate as revealed by studies in experimental morphogenesis. In the study of cellular differentiation at the morphological level, one of our most striking criteria of differentiation has been the appearance of the melanin pigments (Willier, 1942, 1948). The appearance of the pigment, however, is generally too late to be of value in revealing basic constitutional differences in morphologically similar cells. It is clearly established, for example, that in the vertebrates, the melanoblasts have their origin in the embryonic neural crest, and migrate, while in an unpigmented form, to various definitive sites. What constitutional factors, however, differentiate the melanoblasts from other cellular derivatives of the neural crest? In beginning this phase of the investigation, then, we ask whether the melanoblast is characterized by the presence of combining groups identical with, or closely related to, either the fully-formed melanins or intermediate products in their synthesis.

In the past, immunological methods have been applied only sporadically to the study of the antigenic properties of the melanins. Results which have been published hitherto are conflicting, largely owing to the failure of earlier

investigators to distinguish between the antigenic capacities of the melanins, *per se*, and the antigenicity of the complex melanin granule. To cite a case in point, Adant (1932) reported that melanins are antigenic when injected into albino rabbits (but not when injected into normally pigmented rabbits). From an examination of the extraction procedures employed, however, it is evident that the results are subject to the afore-mentioned criticism, for the preparations were undoubtedly of a mixture of complex granules. Therefore, conclusions concerning the antigenicity of the melanins were not justified.

In the present investigation, started in collaboration with S. H. Goodgal, it was first established that pure melanins, prepared by the autoxidation of L-dopa (L-dihydroxyphenylalanine), were not antigenic in either chickens or rabbits, even though the normal sera of adult chickens contain a natural agglutinin for the melanins (Ebert and Goodgal, 1949; Ebert, unpublished results). Repeated injections of dopa melanin into chickens did not raise the agglutinin titer. Consequently, in order to study the antigenicity of the melanins, it has been necessary to couple the pigment to a crystalline or highly purified protein, *i.e.*, to form a melano-protein complex. The complex formation has been carried out by the oxidation of the protein by the enzyme tyrosinase in the presence of a phenolic activator. In this manner, the tyrosyl groups of the protein (and secondarily, possibly primary amino and sulfhydryl groups, as well) are oxidized (Sizer and Brindley, 1950), producing a suspension of pigment-protein. Three melano-protein complexes were prepared: melano-gamma globulin, melano-ovalbumin, and melano-fibrinogen. In a typical experiment, 25 mg. of bovine gamma globulin (Armour) were incubated with 0.5 mg. L-dopa and 0.5 ml. Tremond tyrosinase (containing 1500 Miller-Dawson units and 1 mg. dry weight per ml.), at 37°C., pH 6.0, for eight hours. The resultant melano-protein was concentrated by centrifugation, washed with several changes of 0.9 per cent NaCl and dispersed in the saline solution by grinding in a Ten Brock tissue-grinder. Four albino rabbits were injected with the melano-protein, each receiving a total of 10 mg. of protein over a period of 14 days. The rabbits were bled ten days after the last injection and the antibody titer of each serum was determined by the precipitin method. Three of the four sera tested contained potent antibodies against the homologous antigen. That the melanin moiety of the complex has elicited specific antibodies is shown by the fact that all three sera retained their high reactivity with the complex after prior absorption with bovine gamma globulin alone. When the antisera are mixed with pure dopa melanin, no visible reaction is observed; yet, after such combination, the sera no longer react with the complete homologous antigen. Obviously, the melanin moiety of the complex exhibits the typical behavior of a hapten. It is not surprising, then, that antisera against melano-gamma globulin cross react with both melano-ovalbumin and melano-fibrinogen. Quantitative studies on the extent of cross-reactivity, using the hapten inhibition method, are now in progress, as are tests of the reactivity of these antisera with black and "red" melanins

from embryonic sources. Technical details of the aforementioned investigation will be reported elsewhere.

*Ontogenetic Change in Antigenic Specificity
of the Organs of the Chick*

It will be of interest to examine next the evidence relating to the sequence of differentiation of tissue-specific antigens in the later chick embryo. At this point, one aim of the discussion, as stated in the introduction, "to point out gaps in our knowledge in the field", may be realized in part. We have no information concerning the differentiation of tissue-specific antigens in the chick embryo for the period between the first appearance of somites (1-6 somites) and 72 hours of incubation. The void is a serious one, because it is during this period that the majority of organ primordia first appear. While our knowledge of the formation of tissue-specific antigens is not abundant for any period of development, we do have a nucleus of suggestive facts for the developmental stages both preceding and following the appearance of discrete organ primordia, but none for the interval during which organogenesis is most actively proceeding, the time which, upon examination, may well prove to be the most interesting and fruitful.

Because of the conflicting results of earlier workers (owing largely to the application of techniques which denature proteins) it is encouraging to report not only that three recent investigations have demonstrated the gradual elaboration of several groups of tissue antigens in the chick embryo, but also that the findings tend toward mutual confirmation and supplementation. The latter fact is especially noteworthy since the results have been obtained by the use of four different techniques. The contribution of Burke, Sullivan, Petersen, and Weed (1944) provides an excellent point of departure, because it has exerted an important catalytic influence in the field, in addition to furnishing valuable data. By precipitin and complement fixation methods, Burke *et al.* determined the time at which several adult tissue antigens could first be detected in the tissues of the chick embryo. The earliest stage examined was 72 hours of incubation. The following results were reported: adult antigens first appear in erythrocytes at 100 hours of incubation; in the lens at 146 hours; in kidney at 220 hours; in brain and gonad at 260 hours. In addition, the findings indicate a change in the antigenic components of both lens and brain during development. Of these, the lens has been investigated more thoroughly. The antigens of 90-hour lens were reactive with an antiserum to 160-hour lens, but not with antisera to 300-hour lens; antigen from 120-hour lens reacted with an antiserum to 300-hour lens, but not with antisera against adult lens; and antigen from 160-hour lens was slightly reactive with antisera against adult lens. It was further stated that the period during which adult lens antiserum exerted a relatively specific effect upon the homologous tissue, *in ovo*, approximated rather closely the time of appearance of adult lens antigens, as demonstrated by serological methods. When adult lens antiserum was placed on the chorio-allantoic membrane of embryos of from 134 to 337 hours of incubation, swelling, vacuolation and partial lysis of the lens and immediately surrounding tissues resulted, but

only in embryos of from 146 to 192 hours. The authors suggest that the absence of effects after 192 hours may be due to hardening of the lens. The reaction occurred within sixteen hours after treatment with 0.3 ml. of anti-serum (1:1 dilution, complement added). When antisera to 160-hour lens were employed, all the recipient embryos died. This result is attributed to a lack of tissue-specificity of the serum. It appears likely that suitable use of absorption procedures might have resolved this point.

Burke *et al.* also presented evidence for the appearance of two saline-soluble antigenic fractions in the developing brain. One antigen occurs in embryos from 160 hours to at least 312 hours, while the second appears at 260 hours and is stated to be the definitive adult antigenic fraction. These findings are less conclusive than those obtained with lens, because the anti-brain sera employed were not highly tissue-specific. Strong cross-reactions were obtained with other tissues, especially with testis and ovary. Critical absorption studies were not reported.

Grunwaldt (1949) studied the effects of antisera against three fractions of newly hatched chicken brain, *viz.*, (1) a saline-soluble fraction, (2) an alcohol-soluble fraction, and (3) an alcohol-insoluble residue, on cellular outgrowth in cultures of 9- and 13-day-old chick embryo spinal cord. The use of spinal cord cultures was justified on the basis of experiments which indicated that cultures of all parts of the central nervous system reacted similarly to the antisera. Antisera against fractions 1 and 3 inhibited outgrowth in cultures of both 9- and 13-day-old cord. Antisera against fraction 2, the alcohol-soluble fraction, affected only 13-day-old cord. Grunwaldt concluded that two different adult antigens were produced during the development of the chick brain; one, an alcohol-insoluble antigen, appears on or before day 9; the second, which is alcohol-soluble, develops between the ninth and thirteenth days of incubation.

In comparing the reports of Burke *et al.* and Grunwaldt, we can consider only the findings with respect to the time at which the saline-soluble, adult brain antigens were detected, *viz.*, eleven and nine days respectively. A possible reason for the discrepancy is found in our previous consideration of the greater sensitivity of the tissue culture assay technique than of serological methods. Moreover, further confirmation of the fact that a saline-soluble antigenic fraction of adult brain is present, not only at nine days, but even in the early blastoderm, was presented by Ebert (1950-1951a), who employed the precipitin technique combined with strict absorption procedures as well as the explantation method (*vide supra*). No evidence was obtained, however, to support the report by Burke *et al.* of a "transitory" saline-soluble antigen. The finding that the development of "common" antigens does not necessarily precede the formation of specific antigens is also of interest. In addition to a brain-specific antigenic fraction, Ebert described two groups of common antigens, namely, antigens common to brain and spleen, and brain and heart, respectively. Although the common antigenic fractions were present in heart and spleen as early as the ninth day, they could not be detected in extracts of brain until after the twelfth day of incubation. These facts suggest that the widespread distribution of

an antigen or combining group does not necessarily indicate that it appears earlier in ontogeny than does an antigen localized to a particular organ.

*An Attempt to Correlate the Ontogeny of Tissue Antigens
with the Development of Specific Physiological
Activity*

Weiss (1947) and Tyler (1947) have focussed attention upon the fundamental problem of specificity in the growth of cellular constituents. It is argued that the mechanism of the process of growth may be viewed as essentially analogous to the mechanism of antibody formation, in which antibody globulin of a specific configuration is produced in response to the incorporation of antigen in the site of synthesis. In normal conditions of growth, the constituents synthesized in a cell would be complementary to configurations at the site of synthesis. Under certain conditions, organ antigen introduced into the cellular environment at a time of active synthesis might serve as an accessory template and thus catalyze the formation of additional homologous antigen. This hypothesis was first subjected to experimental test by Weiss and Wang (in Weiss, 1947), who reported that the incorporation of liver fragments from six-day-old chick embryos into the area vasculosa of four-day-old chick hosts elicited a striking response (enlargement) in the growth of the hosts' livers. This enlargement was reflected in cellular hypertrophy and in increased mitotic rate.

At the outset, the present investigation had as its objective "to determine the specificity and time of origin of the substances concerned in the enlargement of the spleen of the host chick embryo following chorioallantoic grafts of adult chicken spleen, and to relate the responsible substances to specific antigenic constituents of the developing spleen." Only a brief summary of the results will be cited here (for details, see Ebert, 1951a). It was first established that the graft substances eliciting a growth response of the host's spleen are class-specific, and quantitatively organ specific. Furthermore, the capacity to evoke enlargement of the host's spleen increased progressively during development. Grafts from embryonic chick donors of less than 14 days incubation proved ineffective, while progressively older donor tissue showed increasing effectiveness. As a further step in the analysis of the factors concerned, the time of appearance of the saline-soluble organ antigens of the spleen was studied, using immunochemical techniques. The results clearly demonstrate that the development of the chick spleen is marked by the synthesis of two groups of specific antigens. The first antigenic group (S_1) has been shown to be present as early as the ninth embryonic day, while the second group (S_2) cannot be detected until after the fourteenth day of incubation. These data suggest a correlation between the progressive increase with age in the ability of spleen grafts to evoke enlargement of the host's spleen, and the differentiation, during a similar period in ontogeny, of a specific group of antigens.

In more recent studies, the serum-agar technique of Oudin (1946, 1947) has been used in combination with the method of reciprocal absorption to determine the minimal number of specific antigens present in each group.

Since the results have been published only in abstract form (Ebert, 1951b), a more detailed presentation is in order at this time. Of the several immunological techniques available for determining the number of antigens present in an antigenic complex, only one method has been described which gives the accuracy and reproducibility needed to approach the problem at hand. The method described by Oudin (*op. cit.*) consists of overlaying a solid immune serum-agar mixture, contained in a thin bore tube, with the antigen solution. An antigen-antibody precipitate is formed as a sharp band which moves down the tube as more and more antigen diffuses into the agar. Only one band is formed when a single antigen-antibody system is present, while multiple systems give multiple bands. The number of bands corresponds to the minimum number of antigen-antibody systems present. The rate at which the band of precipitate moves is proportional to the square root of the time. The potential importance of the technique has led to its detailed comparison with the method of supernatant analysis, by Munoz and Becker (1950, 1951). These authors, using mixtures of antigens and antisera of known immunological composition, have substantiated Oudin's conclusions, and, in addition, have described a method for the resolution of two bands of precipitate migrating at nearly the same rate.

Cooper (1950) has employed the technique in the study of the number of antigens in the egg and early embryonic stages of the frog which are represented by substances with immunologically related combining groups in adult frog's serum.

The serum-agar technique offers a large number of possible applications. In the present investigation, it has been employed for the enumeration of the minimum number of antigens present in antigenic groups S_1 and S_2 of the developing chick spleen, and in effecting the purification of the antigenic components.

Preparation of Antigens; Antibody Production and Titration. White Leghorn chick embryos, incubated for 18 days at 37.5°C ., were used as the source of spleen antigens for injections. Embryos of 9, 12, and 18 days' incubation provided antigens for absorption and test purposes. A total of over 1600 embryo spleens was employed. The only deviation from the procedure previously described (Ebert, 1951a) was in the method of bleeding the donors prior to sacrifice. Blood was collected from the large chorio-allantoic vessels of each embryo by means of a glass canula and refrigerated for later use in absorption tests. The entire preparation was carried out at 4°C . All injections, absorptions, and immunological tests were made with freshly extracted antigens, since previous experiments had revealed frozen and lyophilized antigens to be less satisfactory.

All injections were made into adult male albino rabbits. Four rabbits were injected with extracts of 18-day-old embryonic spleen. Injection schedules followed those previously used for the preparation of antisera to extracts of adult chicken spleen. The animals were bled either seven or ten days following the last injection, and their sera collected in the usual manner. All sera were sterilized by passage through a Seitz filter and stored in a deep freeze at -15°C . Prior to use in serological tests, all sera, both normal

(control) and experimental were treated as follows: (1) heated to 56°C. for 30 minutes to inactivate complement; and (2) completely absorbed with whole blood (pooled from ten 18-day-old embryos). The absorption technique, for absorption with both whole blood and heterologous organ extracts, followed that used in earlier experiments. Preliminary determinations of the organ specificity and of the effective titer with homologous antigen of each serum were made by the precipitin method. A preliminary titration was made with a constant volume of full-strength serum and progressively decreasing amounts of antigen. The end point of this antigen-dilution series was then used with serial dilutions of serum. In this manner, inhibition due to excess antigen is avoided, the serum end-point titer being greatest with the least effective amount of antigen (Ebert, 1951a; Kabat and Mayer, 1948; Martin, 1943). The sera employed were found to be quantitatively organ-specific, confirming earlier results. The serum-dilution titers (reciprocal of highest effective serum dilution) of the four antisera tested with homologous antigen were: SE-1=512; SE-2=512; SE-3=1024; SE-4=512. The highest titer obtained with any heterologous organ antigen was 64, that being with extracts of 18-day-old embryo chick brain.

Serum-agar Technique. The method of Oudin, as modified by Munoz and Becker (1950, p. 48), was closely followed. The following minor variations should be noted: Ten-cm.-lengths of glass tubing with an inside diameter of five mm. were used to make the tubes. The pH of the agar was adjusted to a point between 7.0 and 7.25. In preparing the serum-agar mixtures, the following relative proportions of the reagents were employed: (1) 4.0 ml. of 0.6 per cent agar, melted, cooled to 48-50°C; (2) 1.6 ml. of merthiolate, 1:1000; (3) 1.6 ml. of 0.15 M NaCl, phosphate buffered at pH 7.4; and (4) 0.8 ml. of serum (control or experimental, absorbed with whole blood plus further absorption as indicated). The required number of tubes for each series was then filled from the freshly-prepared stock mixtures. In this manner, greater accuracy was obtained than by mixing the reagents for each tube separately. Thus, a final serum concentration of 1:10 was employed. It is essential that these factors, the concentration of agar, and the total serum concentration, be maintained constant, in order to obtain comparable results from one experiment to another. A third factor, the concentration of antigen relative to that of the antibody, must also be kept constant. Therefore, by preliminary experiments, keeping the antibody concentration constant and varying the concentration of antigen, the most favorable concentration of antigen, namely a 1:100 dilution of the stock antigen, was determined, that concentration being used throughout the remainder of the investigation.

The various serum-agar combinations were overlaid with the antigen solutions to be tested. The tubes were sealed with plasticene to prevent evaporation, and incubated at 25°C for two weeks. The distance of the antigen-serum-agar meniscus from the top of the tube, as well as that of the leading edge of each band of precipitate from the same point was measured, employing calipers accurate to 0.1 mm. Measurements were taken at one-, two-, or five-day intervals as indicated.

Results. Antisera against saline extracts of 18-day-old embryo chick spleen were first tested with saline extracts of 18- and 12-day-old chick spleen by the serum-agar method. In each tube, series of bands of precipitate formed, which moved down the tube as time elapsed. Usually, the bands became more elongated with time. The precipitate within each zone was often more dense at the lower end of that zone, *i.e.*, at the leading border of the zone, than at the trailing border, but this phenomenon was by no means invariably observed. Controls, consisting of antigens diffused into agar columns containing normal serum, were negative. In no case was a zone of precipitate observed.

The results of the first series of experiments are summarized in TABLE 1. The first three days of the experiment were marked by the formation of new zones. The maximum number of zones was usually reached by the fourth day. In a few cases, new zones appeared on the fifth day of the experiment, but none appeared after that time. Changes in size and density within zones occurred, however, between the fifth and tenth days. The data presented show the number of zones and the migration of each zone, in millimeters, after five days (120 to 122 hours). Each value represents the average of at least three separate experiments. The findings clearly show the least number of specific antigens present in extracts of 18-day-old chick spleen to be six. In one series of experiments, however, only five bands were obtained. This latter result emphasizes the fact that the number of bands is equal to or less than the number of systems present. Of the six antigens present in extracts of 18-day-old spleen, only three could be detected in extracts of twelve-day-old spleen. It is indicated, therefore, that, in the period between the twelfth and eighteenth days of development, at least three separate antigens (antigenic group S_2) make their appearance.

This conclusion is confirmed by the results of further studies in which antisera against 18-day-old embryo chick spleen were absorbed with extracts of twelve-day-spleen prior to their titration with homologous antigen by the serum-agar method. The data are presented in TABLE 2, which, again, is a compilation of observations made after five days. Prior absorption of anti-18-day spleen serum with extracts of twelve-day spleen removes specific antibodies against the three components of the S_1 antigenic group, leaving only the three antigens of the S_2 group.

The question now arises as to which of the six bands of precipitate formed in the reaction between the antiserum and its homologous antibody represent antigens of the S_1 group, and which bands are formed by S_2 antigens. One might suspect that the two forward bands of precipitate are formed by the action of S_1 antigens with their specific antibodies because (1) in reactions involving both 18-day and 12-day spleen extracts, the two forward bands migrate at approximately the same rate (distance migrated at five days = 59.1, 59.8, 58.8; and 58.9, 60.5 and 59.7, respectively, in the case of the leading band); and (2) absorption with 12-day spleen extracts removes the bands migrating at those rates. At present, however, the available facts support little more than a working hypothesis, because sufficient quantitative data are not yet available to merit a conclusion. The picture

is complicated by the fact that a similar correlation is impossible for the third antigen of the S_1 group. The trailing band of precipitate in tests employing extracts of twelve-day spleen has migrated approximately 30 mm. by the fifth day (29.3, 29.5, 30.4); yet after absorption of antisera with extracts of 12-day spleen, the reactions of the absorbed sera with extracts of

TABLE 1
SERUM-AGAR PRECIPITIN TESTS

(ANTISERA AGAINST SALINE EXTRACTS OF 18-DAY-OLD EMBRYO CHICK SPLEEN TESTED WITH SALINE EXTRACTS OF 12- AND 18-DAY-OLD EMBRYO CHICK SPLEEN)

<i>Antiserum*</i>	<i>Antigen</i>	<i>Number of bands (after 5 days)</i>	<i>Migration of each band† in millimeters (after 5 days)</i>
ES-1	12-day	3	29.3 40.6 58.9
	18-day	5	13.7 18.7 31.5 41.8 59.1
ES-2	12-day	3	29.5 40.8 60.5
	18-day	6	11.0 16.3 22.6 30.1 40.1 58.8
ES-3	12-day	3	30.4 42.4 59.7
	18-day	6	9.7 16.0 23.1 30.7 41.0 59.8

* All sera absorbed with whole blood; complement inactivated. Control sera were routinely negative.

† Each value is the average from at least three experiments.

18-day spleen produce a band in a similar position at five days (28.5, 30.0, 34.0). These data require supplementation; more comprehensive studies are now in progress.

Attempts at Purification of S_1 and S_2 Antigens. A second feature of the serum-agar method, in addition to its ability to give a measure of the number of antigens present in a complex mixture, lies in the fact that the formation of bands of precipitate effects a clear-cut demarcation of each antigen-antibody system, and hence renders it susceptible to chemical analysis.

Obviously, our interest lies in the chemistry of the antigens, with particular reference to those of the S₂ group. The immediate problem, then, is the separation of the antigen of each band from its specific antibody, in order to have available the purified antigen for (1) chemical analysis, and (2) tests of its activity in eliciting growth of the chick embryo spleen. Previous attempts at splitting the antigen-antibody complex have been successful from the standpoint of purification of the antibody. In general, however, the procedures followed in purification of antibody lead to a denaturation of the antigen, which, moreover, cannot be recovered in a purified state. Our first efforts to split the antigen-antibody complex by techniques sufficiently mild to allow recovery of the purified antigen have not met with

TABLE 2

SERUM-AGAR PRECIPITIN TESTS COMBINED WITH ABSORPTION

ANTI-18-DAY SPLEEN SERA ABSORBED WITH EXTRACTS OF 12-DAY-OLD EMBRYO SPLEEN
PRIOR TO SERUM-AGAR TESTS WITH 12- AND 18-DAY ANTIGENS

<i>Antiserum</i>	<i>Antigen</i>	<i>Number of bands (after 5 days)</i>	<i>Migration of each band in millimeters (after 5 days)</i>
ES-1	12-day	0	—
	18-day	3	14.9 23.0 28.5
ES-2	12-day	0	—
	18-day	3	17.1 25.0 30.0
ES-3	12-day	0	—
	18-day	3	15.0 21.5 34.0

success. In the course of one series of experiments, however, a chance observation concerning the ease of separation of purified antibody from the complex has offered a clue concerning the nature of two of the bands of precipitate. These experiments may be briefly summarized. The technique utilized is based upon the removal of antigen from the antigenic mixture by specific combination with antibody, followed by dissociation of the antibody from the precipitate by strong salt solution. The method, first described by Heidelberger and Kendall (see Kabat and Mayer, 1948), is applicable to carbohydrate-anticarbohydrate systems. It cannot be used for protein-antiprotein systems. The antibody from 15 ml. of antiserum against extracts of 18-day-old embryo chick spleen was precipitated by the addition of 15 ml. of homologous antigen diluted so as to leave a slight antibody excess. The mixture was allowed to stand in the cold for eight hours, after which time the precipitate was collected by centrifugation and washed seven times in 0.15 M NaCl. The washed precipitate was then extracted twice for one

hour at 37.5°C with 30 ml. of 15 per cent NaCl. The 15 per cent NaCl extracts were dialyzed in the cold under negative pressure against repeated changes of 0.9 per cent NaCl and concentrated to a final volume of 5.0 ml. The extracts prepared in this manner, then, should contain purified antibody only if one or more of the six antigens present in the 18-day embryo spleen are carbohydrate. To test this possibility, the 15 per cent NaCl extracts were employed as antisera in serum-agar tests with both 18- and 12-day-old embryo spleen antigens. Two bands of precipitate appeared in each case. It may be inferred, therefore, pending verification, that two antigens of the S₁ group may be carbohydrate in nature, or that they carry carbohydrate determinant groups. The results of these experiments also provide additional confirmation of the findings of Oudin and Munoz and Becker, who observed that the movement of the bands is proportional to the square root of the time. The data are presented in TABLE 3. Each value is the mean

TABLE 3
SERUM-AGAR PRECIPITIN TESTS*

Antigen	Number of bands		Daily migration of bands in mm.†				
			Day				
			1	2	4	6	8
12-day spleen	2	leading trailing	21.7	28.9	42.4	52.4	59.9
			14.7	19.3	29.3	34.9	41.2
18-day spleen	2	leading trailing	22.1	30.5	43.1	53.5	60.4
			16.1	22.6	31.9	38.9	45.4

* In all tests, the serum employed is the "purified antibody" obtained by extracting the precipitate formed by the reaction between anti-18 day embryo chick spleen and its homologous antigens with 15 per cent NaCl. Details in text.

† Each value is the mean from six trials.

of at least six experiments. Additional experiments in which the washed antigen-antibody precipitates were extracted by a procedure employing strong salt solutions followed by ether (Kabat and Mayer, p. 481) indicate that Wassermann-type antigens are absent from the spleen extracts studied.

Although the experiments outlined above are of a preliminary nature and several technical obstacles still need to be surmounted, it appears quite clear that the use of the serum-agar method combined with chemical analysis offers great promise as a critical tool for embryologists interested in the question of the time and manner of origin of specific proteins. It may reasonably be expected that this tool will prove to be indispensable in studies of substances in the embryo otherwise not detectable.

General Conclusion

The evidence at hand justifies only the conclusion that quantitative and qualitative differences in tissue-specific protein and conjugated protein molecules occur during ontogeny. In this case, ontogeny is used in the broad sense, including post-embryonic development. Certain tissue-specific anti-

gens are present in the chick embryo well in advance of the appearance of the corresponding organ primordia. Other antigenic substances can be detected only after histogenesis is well underway, yet not all molecular species can be detected at any period during embryonic life, appearing only after hatching. Moreover, it is indicated that the development of the chick is marked by the differentiation of transitory, embryo-specific antigens, in addition to the tissue-specific antigens characteristic of the adult. The data on this point require supplementation, however. Although it is clear that development is characterized by progressive change in complement of proteins and other complex molecules, it has not yet been determined to what extent the observed changes reflect true synthetic processes and not the separation of complex molecular associations, resulting in altered antigenicity. Moreover, it has not been demonstrated that such changes in protein molecular composition play a causal role in morphogenesis. While some progress has been made in efforts to associate the appearance of a tissue-specific antigen, or group of antigens, with a specific physiological role in development (Ebert, 1951a, b), and to link alterations in protein structure with discrete morphological changes (Ranzi, 1951), sufficient data are not yet available to establish such correlations. A few questions have been answered in part. The time at which several specific antigens can first be detected with the methods at hand can be stated with some assurance, yet the whole problem of the manner of origin and role in morphogenesis of tissue-specific proteins remains unsettled. The field is still young. More critical results yet may be expected.

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PHYSICAL AND CHEMICAL CHANGES IN THE CIRCULATING BLOOD

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The circulating blood of the embryonic chick differs, in some respects radically, from that of the mature organism. Its peculiar or characteristic features are especially worthy of consideration since we must suppose it is adapted to serve the needs of rapidly-growing and differentiating tissues. From this point of view, its constitution and properties are of interest for the *in vitro* culture of tissues, the propagation of microorganisms and normal and malignant tissues, as well as the physiology of development.

The morphological aspects of the blood are perhaps best known, although the basic genetic relationships between the various cells and the causal factors of their differentiation are still obscure. At the present time, there is especial need of quantitative studies of the cellular elements and their variations under the influence of specific infectious agents, foreign tissues, and various chemical and physical factors. Such studies will permit the more extensive use of the chick embryo as a convenient test organism in many situations. The non-cellular portion of the circulating blood, however, is even less known than the cellular portion, and, in the following text, we shall be concerned mainly with several aspects of the serum which present distinct quantitative or qualitative differences during the course of development.

The serum of the early chick (3-13 days incubation)* impresses one as a very watery fluid and this is borne out by studies of the total solids¹ (FIGURE 1). The ten-day serum contains only 40-45 per cent of the solids in adult serum, taking 65 mg./ml. as an average value for the adult fasted bird. The average value for the 10-day serum is 28 mg./ml. (range, 26.5 to 30.8 mg./ml.). This value has not changed perceptibly by the 13th day, which suggests the possibility that this value may be characteristic of all the earlier stages of development. Serological evidence discussed subsequently suggests, however, that this is unlikely and that a marked increase in serum proteins probably occurs during the fourth to the eighth days of development.

From the 13th day of incubation to hatching, the total serum solids increase to almost double the value of the ten-day serum. On the other hand, the post-hatching chick shows little or no increase until sometime during the second month of life.¹

Although total serum lipid is relatively high during the entire embryonic period, its striking predominance becomes apparent when it is compared with the defatted solids which are mainly protein (FIGURE 1). Protein increases at a moderate rate up to the 19th day and then more rapidly. It approximately doubles in concentration between the tenth and 19th days

* The eggs used in our own studies have been almost exclusively the New Hampshire breed, and are incubated in forced draft incubators at 37°C.

and again between the 19th and 22nd day (one day after hatching). The lipid curve starts upward a few days after the protein increase, and begins to decline shortly after the protein starts its rapid climb near the end of the embryonic period.¹ Vladimiroff and Schmidt²² noted a similar lipid trend but recorded maximal values at the 17-18th days, probably because their eggs were incubated at a higher temperature. Phospholipid is apparently an important constituent of the total lipid, since there is a high lipid phosphorus/protein nitrogen ratio in the serum of the 13- and 15-day embryo.⁶ A large fraction of the phospholipid is evidently carried by the faster electro-

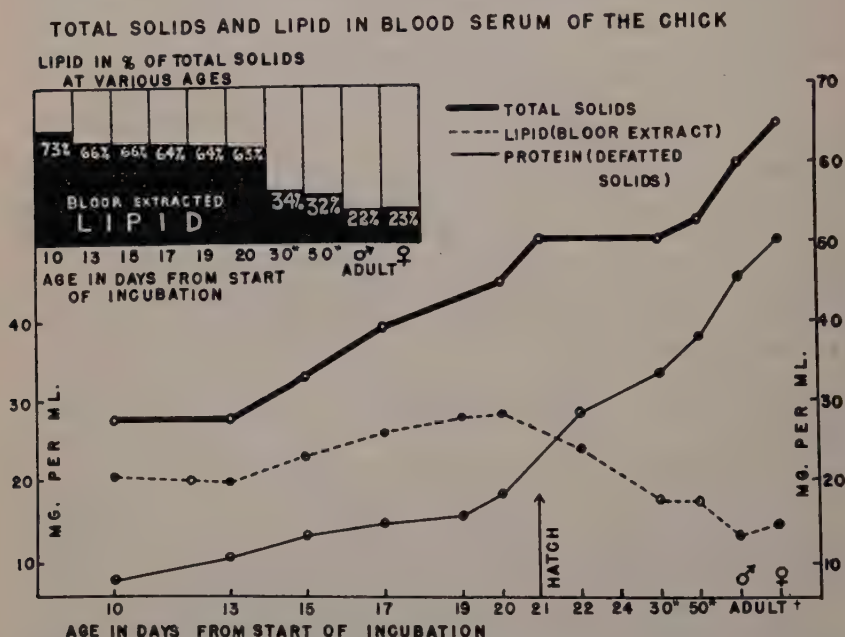


FIGURE 1. Total solids, Blood-extractable material, and defatted solids. (*) Starred ages represent days after hatching. (†) Adult values based on sera from birds fasted 24-48 hrs.

phoretic components of the serum, the latter having an unusually high lipid phosphorus/protein nitrogen ratio.⁶ Blood cholesterol also increases to a high on the 18th day and then drops.²¹ It is of interest that the cholesterol curve may form a plateau between the tenth and 13th days²¹ corresponding to the plateau we observed in the total lipid. In the several days following hatching, however, the cholesterol increases again,²¹ whereas the total lipid maintains the steady decline begun in the embryonic period.¹ This post-hatching cholesterol rise must be transient since total blood lipid and its constituents (cholesterol, fatty acids, and phospholipid) declines after the third day post hatching.²

The distinctive constitution of the embryonic serum is apparent when the lipid is expressed as percentage of total solids (inset, FIGURE 1). In the ten-day chick, the serum solids are approximately three-fourths lipid and

one-fourth protein. Lipid predominance declines moderately between the tenth and 13th days and then very gradually until hatching. Within a day after hatching, the relationship has reversed and the protein dominance characteristic of post-hatching life sets in. Finally, in the adult fasted bird, the lipid/protein ratio is essentially opposite that found in the ten-day embryo.

The refractive index of the serum during development is a useful means of estimating approximate concentrations (FIGURE 2), but the changing constitution of the serum introduces some deviations. The refractive index, how-

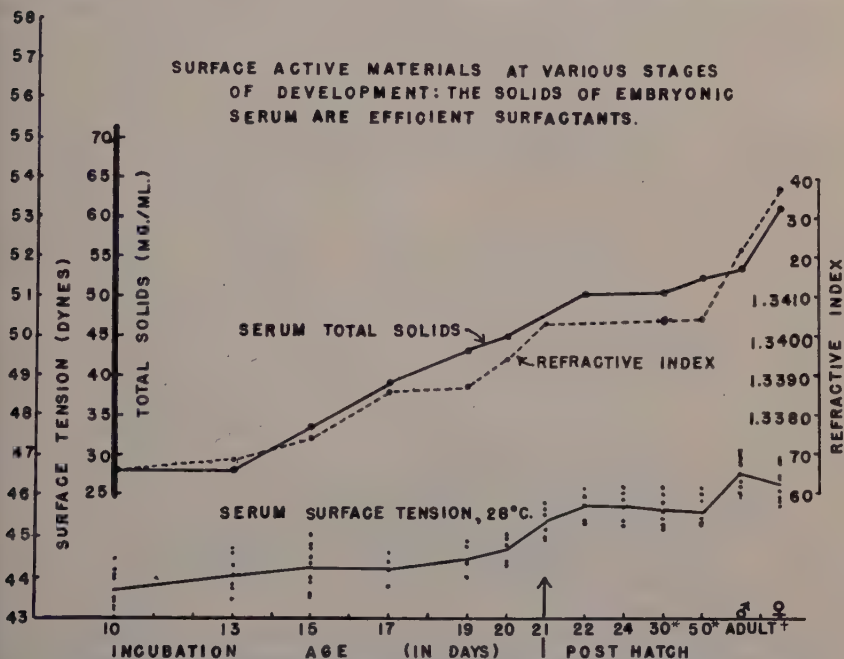


FIGURE 2. Surface tension during development (Du Nouy tensiometer). Each dot represents equilibrium surface tension. Readings taken at about two-minute-intervals. Temperature, 28°C.

ever, can be used to demonstrate qualitative differences between embryonic and adult sera. This demonstration is based on the principle that, while the various protein constituents differ slightly in their refractive increments, lipoproteins have a markedly lower increment.³ It will be noted in TABLE 1 that the samples of 13-day serum contained the same total solids as the 10-day serum, but a larger proportion of the solids is protein (FIGURE 1). The higher refractive increment of the 13-day serum is in line with its higher protein/lipid ratio.

Although the nutriment of the embryo (egg yolk) is rich in lipid, the high lipid content of the serum does not represent a lipemia due to chylomicrons easily separable by strong centrifugation. The ten-day serum may be centrifuged in the cold at 15,000 Xg for 30 minutes without detaching lipid as a separate layer.⁷ On the other hand, a floating layer rich in lipid phospho-

rus is obtained by increasing the specific gravity of the serum with NaCl and centrifuging for three to four hours.⁶ After such treatment, the faster electrophoretic components (components 1, 2, 3 of FIGURE 6; components 7, 8, 9 of Marshall and Deutsch) are markedly decreased.⁶ We have observed the presence of fractions of low specific gravity during the course of ammonium sulfate fractionation of the ten-day serum.¹⁵ Much of the protein precipitated at 33-48 per cent ammonium sulfate saturation floats in this salt concentration, whereas the corresponding fraction of adult laying-hen serum is thrown to the bottom of the tube. These observations suggest that the embryonic serum contains a higher proportion of lipoproteins than is found in adult sera.

The surface tensions of embryonic sera (as determined with the Du Nouy

TABLE 1
REFRACTIVE INDICES OF SEVERAL SAMPLES OF EMBRYONIC AND ADULT SERA*

<i>Serum</i>	<i>Refractive index</i>
10-day embryo	1.3364
	1.3365
	1.3367
	1.3367
13-day embryo	1.3371
	1.3371
	1.3367
	1.3371
adult fasted hen (diluted)	1.3390
	1.3383
	1.3384
adult cock (diluted)	1.3388

* Diluted with physiological saline to contain the same concentrations of total solids.

tensiometer) are low, but differ from the adult serum values by only 2-3 dynes/cm. after the surface has matured to constant values¹ (FIGURE 2). If we consider the total solids, however, it is apparent that the embryonic solids are far more efficient surfactants, the efficiency increasing with decreasing age. The average surface tension of ten-day serum is about 43.5 dynes/cm. (range: 43.0-44.5). Adult sera diluted to contain the same total solids as the ten-day serum give surface tension values ranging between 47.6 and 52 dynes/cm.¹

Surface tensions of various embryonic and adult sera, as a function of time, are shown in FIGURE 3.¹ Post-hatching and adult sera show a gradual decrease in surface tension with time, a phenomenon characteristic of many colloidal surface-active materials. Adult male serum is outstanding in this respect. Embryonic sera, on the other hand, show essentially constant surface tensions. It is possible that a drop occurs during the two or three minutes commonly required to obtain the first reading. In any event, the surfaces of embryonic sera mature with great rapidity. Since proteins probably undergo denaturation at interfaces,³ it is probable that embryonic sera

contain specific labile constituents or a higher proportion of such constituents than do the post-hatching stages. This supports other evidence that lipoproteins constitute a relatively important portion of the embryonic proteins, because lipoproteins are unstable in many respects.^{3, 23}

The hypothesis of labile embryonic constituents rapidly denatured at interfaces is supported by observations of interfacial tensions between sera and xylene-bromobenzene mixtures¹ such as are commonly used for estimating the specific gravity by the falling-drop method. The ten-day serum

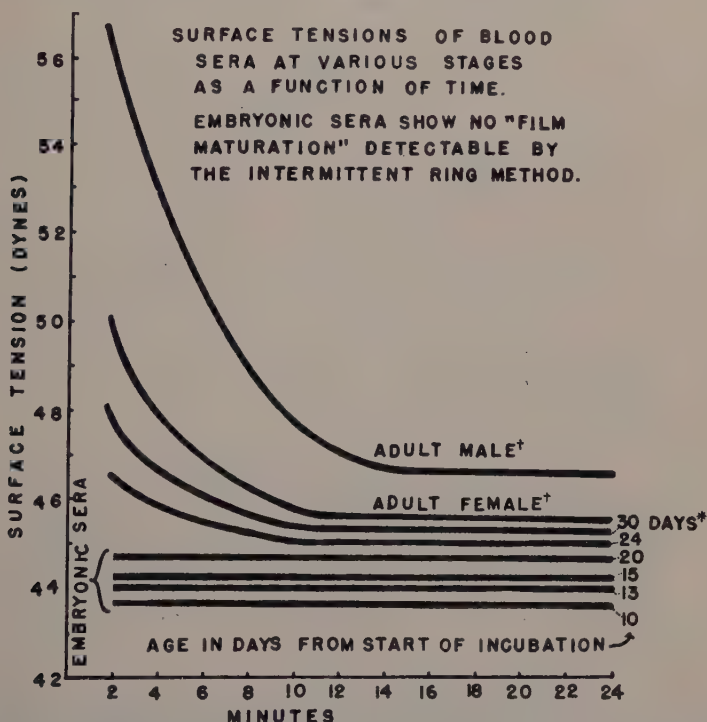


FIGURE 3. Surface tensions of sera as a function of time. Temperature, 28°C. (*) Days after hatching. (†) Birds fasted 24-48 hours.

suspended as a hanging drop in the xylene-bromobenzene at 20°C shows an extremely rapid decrease in interfacial tension, so that the drop breaks into two spheres. The advanced embryonic, post-hatching, and adult sera maintain interfacial tensions of sufficient magnitude to preserve the mass of the original drop until it is coagulated.

Preliminary studies on the serum of advanced rabbit fetuses indicate that their surface tension is generally lower than that of the mother or other adult animals and that the lipid/protein ratio is higher.²⁴ Our observations on the chick are therefore possibly of general application to higher vertebrates and are not dependent upon the conditions of life imposed by the cleidoic egg.

The instability of embryonic sera is an important consideration in its

storage. Lyophilization of the ten-day embryo serum (rapid freezing in dry ice-acetone and dessication from the frozen state *in vacuo*) is not a recommended procedure if reconstitution is contemplated. The solids re-dissolve to a limited extent, forming a milky suspension which separates on centrifugation into a whitish floating pellicle and a heavier, sedimentable fraction. Adult sera subjected to an identical procedure are readily reconstituted. Storage of ten-day serum in the frozen state results in less obvious denaturation but this method also must be regarded with caution in view of the instability of lipoproteins to freezing and thawing.³

The instability of embryonic sera, especially in the earliest embryos studied (10-14 days incubation), is shown in a striking manner by studies of heat denaturation. Although low in total solids, the 10-14 day sera form dense white precipitates, in contrast to the later embryonic and adult sera. This may be demonstrated rapidly by heating equal volumes of the various sera in a boiling water bath for 2-3 minutes. Under these conditions, the ten-day serum is converted to an opaque white emulsion, whereas only slight cloudiness appears in the advanced embryonic sera and in adult sera. FIGURE 4 shows relative amounts of precipitate as measured nephelometrically at $\frac{1}{2}$ minute intervals at 85°C.⁴ Since there is the possibility that the 10-14 day sera are heat-labile merely because their protein concentrations are lower, the advanced sera were diluted to various degrees with veronal buffer, pH 8.6. Such diluted sera still form markedly less precipitate than the early sera (see 16-day serum, FIGURE 4).

The declining heat-lability of embryonic sera at several temperatures and dilutions is shown in FIGURE 5. All samples were heated for an equal period (ten minutes). The precipitation curves decline during the course of development to minimal levels near the end of the embryonic period. The unusually high nephelos readings obtained with some adult sera (see GEL, FIGURE 5) are an anomaly for which we have no explanation. Such high nephelos values may be related to gel-formation which occurs in the adult but not in the embryonic sera.

The distinct constitution of embryonic sera is shown by electrophoretic studies.^{5, 6} Some patterns obtained in our laboratory are shown in FIGURES 6 and 7.⁷ The identity of the embryonic components which correspond, at least in mobility with the adult serum components, still may be in doubt. Moore *et al.*⁵ found that the fastest component most closely approximates the mobility of adult albumin, and that the main mass of the ten-day serum proteins (components 3, 4, FIGURE 6) have the mobility of adult β -globulins. Marshall and Deutsch,⁶ on the other hand, find that about 50 per cent of the ten-day proteins (corresponding to components 1, 2, and 3 of FIGURE 6) have a greater mobility than that of adult albumin and disappear from the serum by the third day after hatching. Tentative estimates of mobilities in our laboratory indicate that the region occupied by components 2-3 (FIGURE 6) corresponds in mobility to adult albumen and region 4 (FIGURE 6) corresponds to the α - β region of the adult.⁷ It must be emphasized that all these investigations agree that the embryonic serum is electrophoretically distinct from that of the post-hatching and adult stages, and that compo-

nents of approximate γ -globulin mobility are present in lower concentrations and constitute a smaller fraction of the total protein.

FIGURE 7 shows the electrophoretic pattern of the 15-day embryo serum and the position occupied by adult albumin added to it.⁷ The albumin was

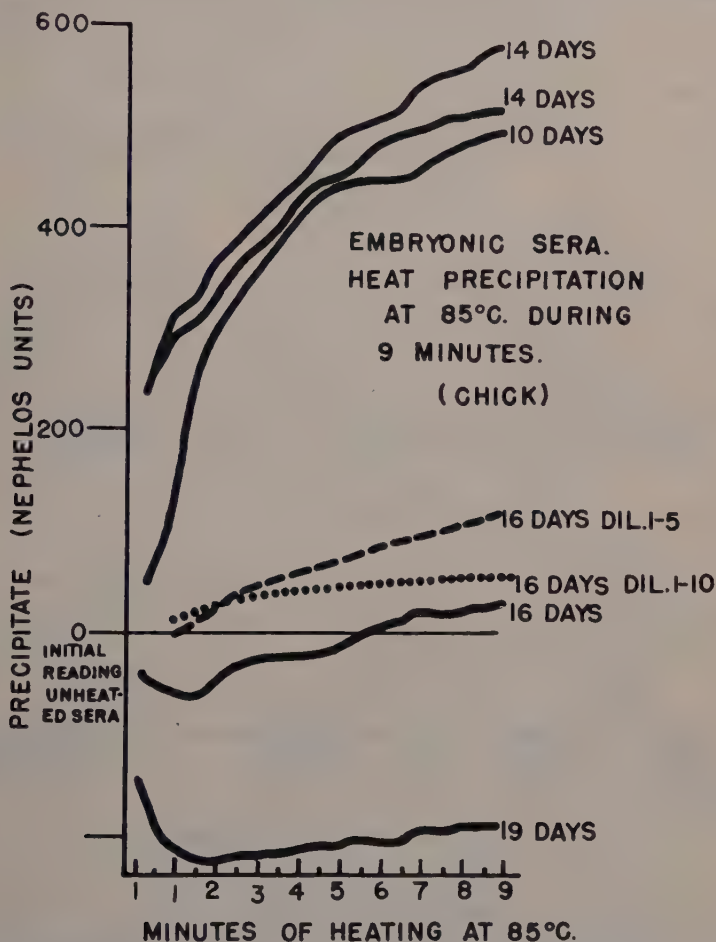


FIGURE 4. Heat denaturation of embryonic sera at 10, 14, 16, and 19 days of incubation. The variation between 10- and 14-day samples are probably not significant. Note that curves for 16-day serum are low in both undilute and diluted samples. Diluent: veronal buffer, pH 8.6.

obtained by the cold ethanol method. This suggests that a large fraction of the embryonic serum has the mobility of the α - β globulins of the adult. A component faster than adult albumin is clearly indicated. This is the component which disappears some time after hatching. An ante-adult component has been found in the serum of the laying hen,^{5, 7, 8} so that this component cannot be considered specific to embryo, even though it disappears after hatching.

Serological studies of embryonic sera, in agreement with the electropho-

retic data, show that the main adult serum constituents are present in the ten-day embryo.^{9, 10} It has been possible to study sera from stages as early as the 3rd day of incubation by the serological method. Anti-adult albumin sera freed of α -, β -, and γ -globulin activity by absorption, react with sera from three- and six-day embryos as well as all later stages. γ -globulin or antigenically similar substances could be detected by the ninth day but not

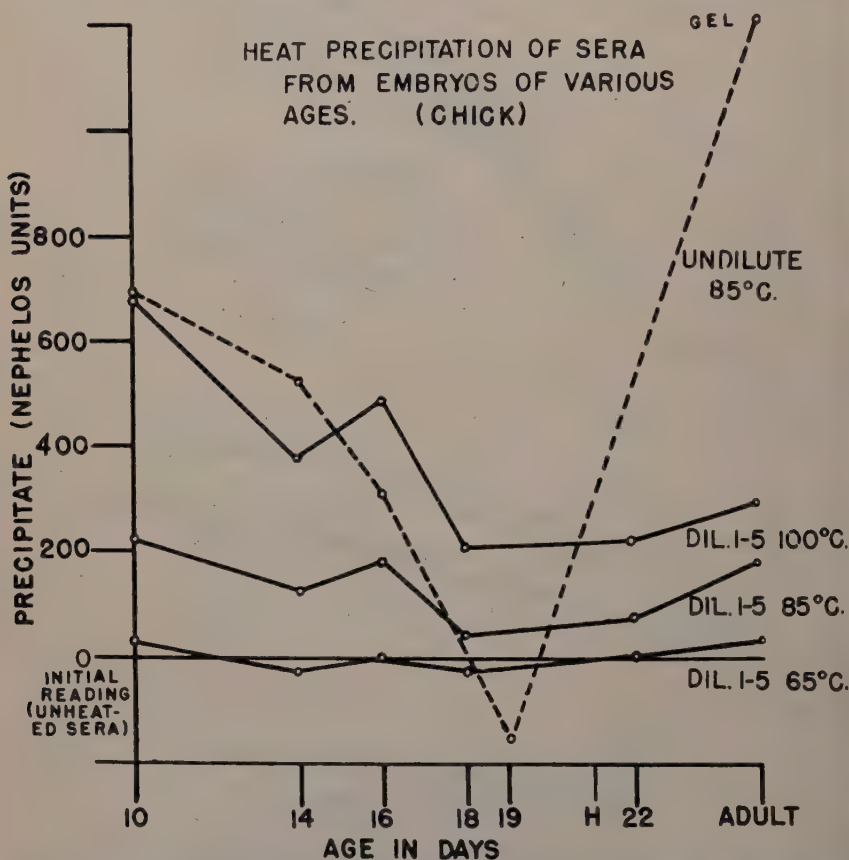


FIGURE 5. Heat denaturation of embryonic sera, undilute and diluted with veronal buffer. GEL indicates that undilute adult sera form gels. All sera heated ten minutes.

earlier.⁹ Antigens or combining groups specific to the α - β globulin of the adult can be detected in the blood from the third day on, and probably in the serum.¹⁰

We have noted in serological studies that antisera too weak to react with three-day embryo blood readily form precipitates with the five- or six-day and later bloods.^{10, 11} This may indicate a quantitative change in antigenic constitution at this time, as was previously suggested, or a quantitative increase to a level which permits weak antisera to form a visible precipitate. Thus, using antisera against the α - β globulin of the adult, it was found that

a given antiserum could react with six-day blood extracts, but not with three-day blood nor with the sera from either stage. This suggests that higher concentrations of antigenic substance are present in the whole blood extracts at the six-day stage and that they are derived from the cellular elements. It is of interest in this regard that a rapid alteration of the blood cell picture takes place during the fourth to the sixth days of development (FIGURE 8). The hemoglobin-rich primitive red cells are at this time replaced by the smaller definitive red cells which contain a lower concentration

BLOOD SERUM OF THE 10 DAY CHICK EMBRYO

ELECTROPHORETIC PATTERNS, VERONAL-NA CL BUFFER, pH 8.35, $\mu = 0.144$

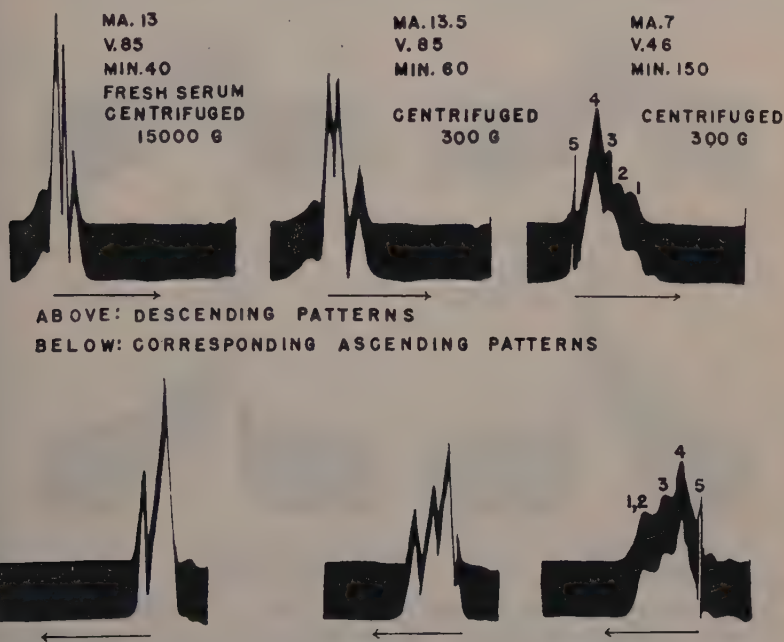


FIGURE 6. Electrophoretic patterns of ten-day embryo serum. MA, milliamperes. V, volts per cm. MIN, duration of experiment in minutes.

of hemoglobin.¹²⁻¹⁴ The percentage of degenerating cells reaches a peak at about the sixth day and then declines. Considered in relation to the serological data, these events suggest that destruction of cellular elements contributes appreciable quantities of protein to the embryonic serum. It may be mentioned, at this point, that FIGURE 8 illustrates one of the difficulties involved in studying the blood picture of the embryo. It will be noted that both Fennel¹⁴ and Sugiyama¹² agree that there is a relatively high proportion of primitive non-hemoglobin cells up to the fifth day of development. These cells are classified as hemocytoblasts by Fennel, as thrombocytes or thromboblats by Sugiyama, and are the same cells formerly called lymphocytes by Dantschakoff. According to Fennel, thrombocytes do not appear until about the twelfth day of development.

Although no constituent like the fetuin of certain mammals²⁵ has been detected as yet in chick serum, serological studies show that the embryonic stages contain antigens not found in the adult serum or plasma. FIGURE 9 shows two methods of absorption used to demonstrate the presence of antigens peculiar to the ten-day serum.¹⁵ Such substances may be termed embryo-specific. The electrophoretic evidence of Marshall and Deutsch¹⁶ show that several fast serum components are lost shortly after hatching, but these may not be embryo-specific since a similar component is present

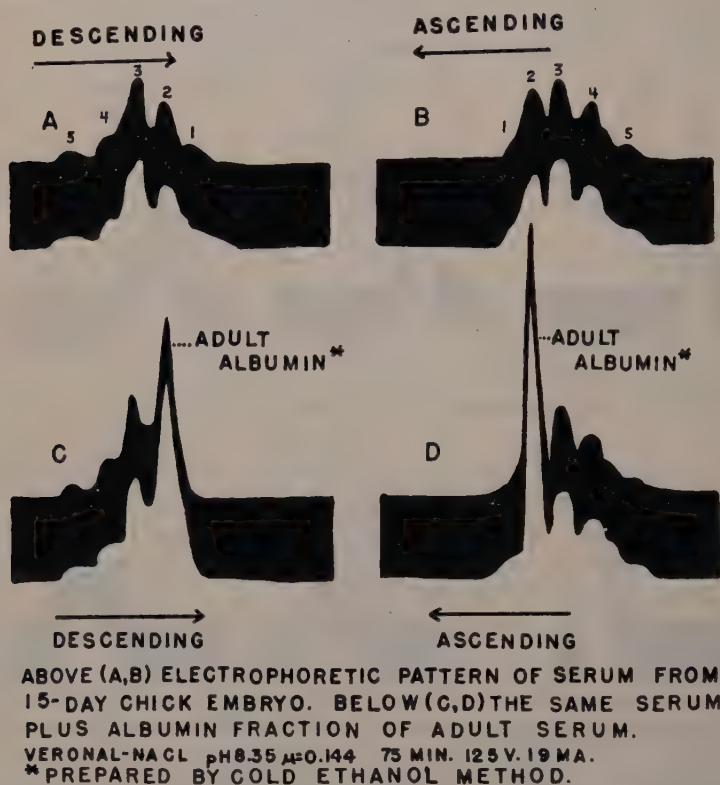


FIGURE 7. Electrophoretic patterns of 15-day embryo serum (above) and the same with added adult albumin.

in the laying hen. It is, nevertheless, of great interest that the embryo-specific antigens disappear at about the same time as the fast electrophoretic components.¹⁵

The antigenic specificity of embryonic serum has also been shown by the quantitative serological method.¹⁶ Rabbit antisera were prepared against adult laying-hen plasma and against the plasma from the ten-day embryo. As expected, the anti-adult sera give higher antigen-antibody precipitation curves with hen serum than with the embryo serum (FIGURE 10). The significance of such curves has been established by numerous immunochemical studies: They indicate two dissimilar antigenic substances or mixtures of substances. An unusual relationship is found when anti-ten-day serum

is reacted with adult and ten-day serum. This antiserum forms higher curves with the heterologous antigen, adult serum. The antiserum is obviously not lacking in antibody since it produces heavy precipitates with adult material. It must be concluded that the embryonic serum forms antigen-antibody complexes with inferior light-scattering properties or that it contains substances inhibitory to the precipitin reaction.

Substances with similar electrophoretic mobilities and serological properties occur in egg white, the 13-day serum, and adult serum. These have been identified as conalbumin and ovalbumin.¹³ Although these proteins are obviously not specific to the embryonic serum, it is of interest that the

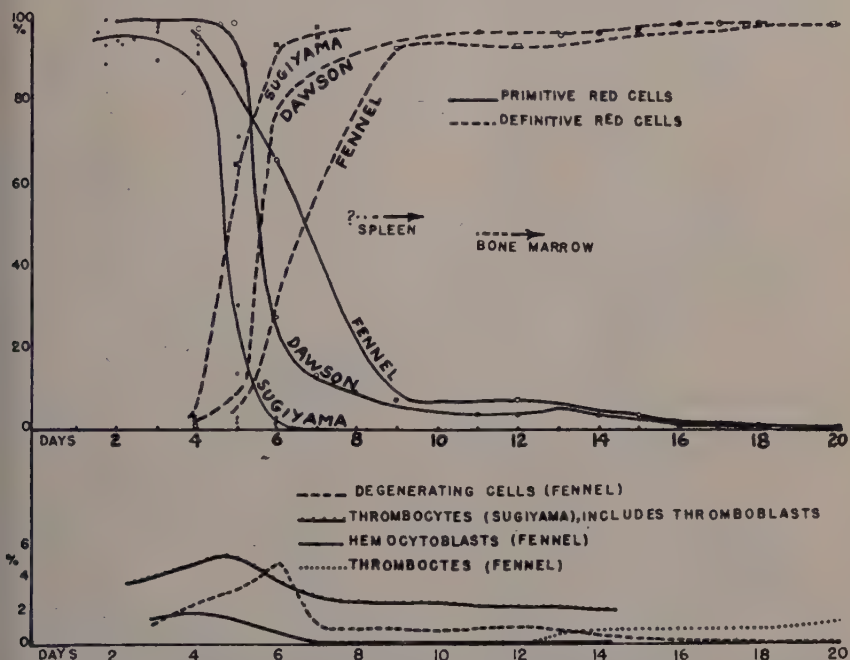
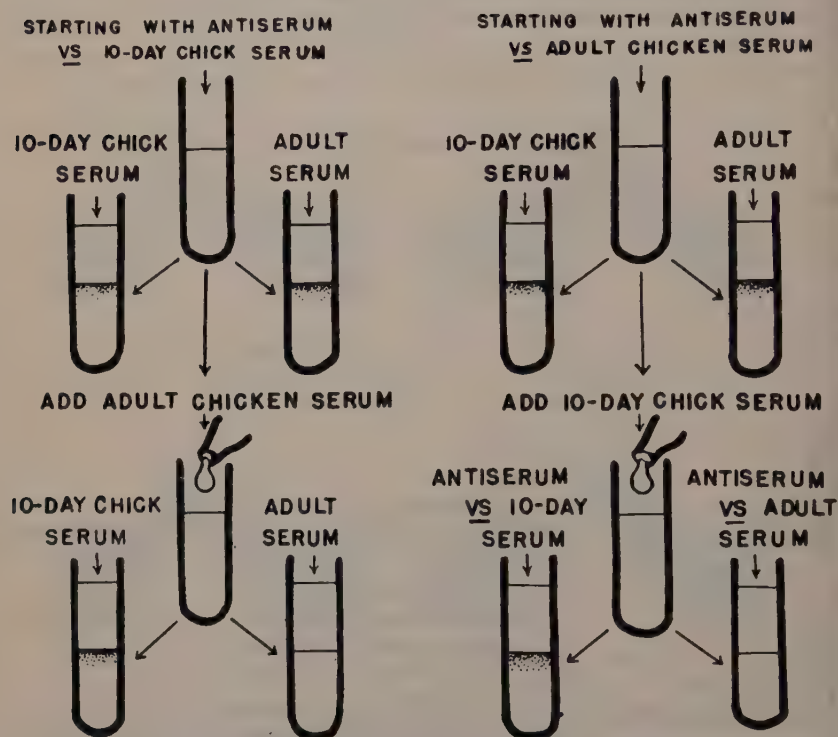


FIGURE 8. The changing blood picture of the chick embryo. Compiled from data of Sugiyama,¹² Dawson,¹ and Fennel.¹⁴ Arrows indicate when hematopoiesis starts in bone marrow and possibly in the spleen.

embryo-specific antigens are similar or identical with substances occurring in the extra-embryonic fluids.¹⁵ This was shown in the following way. Anti-ten-day serum was made embryo-specific by absorption with adult serum. The serum reacted with extra-embryonic fluids (a mixture of egg white and chorio-allantoic fluid). After absorption with the latter, the anti-embryonic serum lost all of its specific anti-embryo activity.

The origin of the embryonic serum constituents, whether they are exclusively of embryonic manufacture or are partly of maternal origin, is an intriguing problem about which we have little data. It has been known for some time that antigens of very similar nature occur in the adult serum, egg yolk and white. Moreover, the embryonic serum contains antigens in common with the egg yolk and white.^{11, 17} However, such data do not, of course, indicate the site of manufacture. The essential evidence for the

presence of proteins of maternal manufacture in the embryonic blood is derived from studies of the transfer of immune bodies from the hen to the yolk or embryo.¹⁹ Work now in progress indicates that rabbit serum or certain of its antigenic constituents can be absorbed from the yolk without loss of species (rabbit) specificity. Rabbit serum is injected into the yolk on the sixth or seventh day of incubation. Seven or eight days later, the



TWO METHODS USED TO SHOW EMBRYO-SPECIFIC ANTIGENS IN SERUM OF THE 10-DAY EMBRYO

FIGURE 9. Diagram showing methods of serological absorption used to demonstrate embryo-specific antigens.

embryos are bled from a chorio-allantoic blood vessel. Using chicken anti-rabbit serum as a reagent, rabbit antigens are demonstrable in the embryonic serum²⁰ in some of the chick embryos. In other embryos, no rabbit antigen is demonstrable, so that the evidence for absorption of proteins of maternal manufacture is inconclusive.

Summary

The blood of the embryonic chick is a system with physical and chemical properties which differ widely from those of the post-embryonic stages of life. The embryonic serum solids are predominantly of lipid character.

The predominance of proteins over lipids is a developmental characteristic attained shortly after hatching. The embryonic serum is characterized by low surface tension and its solids are relatively efficient surfactants. It is a relatively labile system as shown by the ease with which certain of its components are denatured at interfaces, by lyophilization, salt precipitation, and heat. It is probable that lipoproteins are more abundant in the embryonic blood than in later life. The electrophoretic components differ quantitatively from those of the adult serum and possibly include fast components

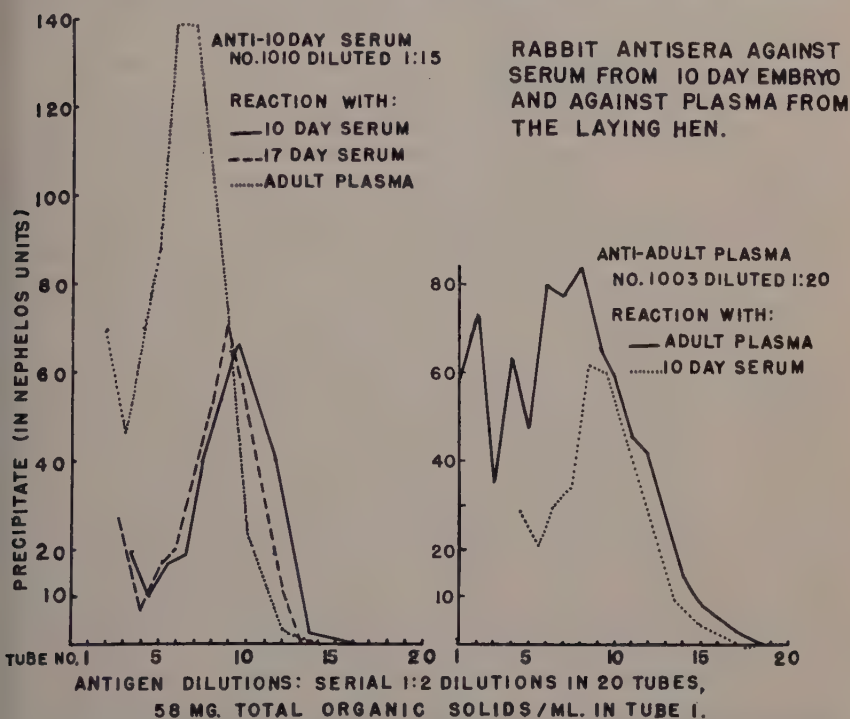


FIGURE 10. Antigen-antibody precipitate curves obtained with rabbit antisera *vs.* ten-day embryo and adult hen sera.

specific to embryonic life. Serologically, the embryonic serum contains some components similar to those of the adult, and others which are limited to the embryonic period. The available evidence supports the view that some of the serum proteins are of maternal manufacture, but this could not be demonstrated after injection of rabbit serum into the yolk.

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STUDIES OF MUSCLE DEVELOPMENT*

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Recent embryological investigations have concerned themselves mainly with the nervous system, the lens, the melanophores or the total limb, while muscle tissue has received only slight attention.

The sporadic studies of muscle development have remained essentially of a descriptive nature (Patten, 1948). Perhaps the only experimental work dealing with one specific aspect of muscle development is the investigation of nerve muscle relationships begun by Harrison (1904) and continued by Hooker (1911), Hunt (1932) and Hamburger (1939).

For a considerable time, the biochemical analysis of development has separated itself more or less from the structural aspects of the developmental process. This is true in studies of various forms of metabolism in embryonic tissue. Another line of biochemical analysis followed closely the approaches indicated by experimental embryology as borne out by the search for the nature of the organizer and the chemical basis of induction (Needham, 1942). Only in the most recent time, an increasing amount of chemical work has been dealing with phases of development which can be tentatively described as cytoplasmic differentiation. The aim is a better understanding of the mechanism by which the rapidly growing undifferentiated cell is transformed into a non-proliferating mature cell with its specific structural and functional properties.

The preceding papers in this meeting are examples of such work. In addition, I should like to point to the studies of Flexner (1950) on the brain of mammals, and the studies on the central nervous system in amphibians by E. J. Boell (1944, 1946).

The main reasons for our interest in muscle development are the recent advances in the biochemistry of muscle tissue of the adult organism (Szent-Györgyi, 1951). The great accomplishment of the work on muscle is the far-reaching explanation of the specific function and structure of the muscle cell by the behavior of certain well-defined proteins. This opened possibilities for the study of embryonic development which were not realized before. By the determination of the specific contractile muscle proteins (actomyosin) one can express, for example, the progress of differentiation in quantitative terms. The rate of the formation of these proteins can be compared with the rate of formation of the unspecific proteins of the rapidly proliferating undifferentiated cell and thus provide clues to possible factors involved in the formation of these two types of cell materials. These considerations, as well as the homogeneity of muscle tissue and the cytoplasmic material of the muscle cell, invite an attempt to give a systematic account of the entire line of development from the precursor of the myoblast to the full maturation of the tissue.

The first series of analyses of developing muscle tissue were carried out

* The support of the work reported in this paper by the American Cancer Society, the Association for the Aid of Crippled Children, and the Boettcher Foundation is gratefully acknowledged.

on rats. The work was then continued with chick embryos, because the early stages are so readily accessible in chicks. We have approached the developmental lineage of the muscle cell from the two opposite ends. On the one hand, we are trying to learn more about the development of the somite. On the other, we are studying the development of the contractile apparatus of the muscle of the chick embryos. Eventually, we hope to learn more about the relation of these two phases of development to each other.

As a first step in the analysis of somite development, we attempted to describe the growth of the somite by the measurement of the increase of several independent magnitudes: the linear dimensions; the volume, which indicates at the same time the wet weight; and three chemical constituents of the somite cells, the protein nitrogen, ribosenucleic acid (RNA) and desoxyribosenucleic acid (DNA) (Herrmann, Schneider, Neukom and Moore, 1951).

The measurements of the linear dimensions of the somites were based on the assumption that the complex shape of the somite could be approximated by a prism. The sides of the prism are represented by the dorsolateral, the medial and the ventral faces of the somite, and the base of the prism, which is facing the adjacent somite. The detailed results of the measurements of the linear dimensions are of no particular relevance in the subsequent discussion and are omitted here. It should be pointed, however, that the linear measurements on individual somites have corroborated older observations of other authors (Williams, 1910-1911), who noted that the size of the somites at the time of their appearance increases with the somite stage. We found that one of the middle somites has the dimensions of $154 \times 148 \times 138 \times 169$ micra shortly after its appearance at the 8-somite stage and $188 \times 193 \times 193 \times 198$ micra at the 22-somite stage. The 20th somite has the dimensions of $202 \times 180 \times 195 \times 186$ already at the time of its appearance at the 20-somite stage. Since the later somites not only grow faster, but also develop more rapidly than the earlier somites, the greater part of the total somite column is in about the same developmental state. These findings are mentioned as a justification for speaking of an average somite.

Accepting the prismatic shape of the somite as a valid approximation, one can use the linear measurements for a calibration of the volume of the somite and, in time, comparison of the calibration data with the linear measurements of the volume indicates to what extent such an approximation is accurate. The data in TABLE 1 show that the calculations and the data obtained by direct measurement are in rather close agreement in reality. The figures also show that the volume of the average somite increases about 26 times. This can also be regarded as an index for the increase in wet weight.

Compared with the increase in volume (wet weight), the protein nitrogen, ribosenucleic acid and desoxyribosenucleic acid, which form the bulk of the dry matter of the somite, show a slower accumulation (15 times, 15 times and 10 times, respectively, for the three substances, see TABLE 2). Also the rate of increase of these substances does not seem to be uniform,

but shows two sharp peaks which occur synchronously for all three substances (FIGURE 1). This suggests a remarkable parallelism in the accumulation of these three chemical cell components, PN, RNA and DNA during somite development.

The data reported so far cover the first five days of the embryonic development. During this time, the somite cells have become segregated into a

TABLE 1
VOLUMES OF AVERAGE SOMITES*

Somite stages	Volume in cubic millimeters per somite for the average of five somite stages		Increase in per cent
	I	II	
16-20	.004	.003	
21-25	.006	.007	50
26-30	.010	.012	67
31-35	.016	.022	60
36-40	.030	.041	87
41-45	.066	.060	122
46-50	.097	.098	45

* Derived (I) from direct determinations of the volumes of whole somite columns (volume of somite column/number of somites) and calculated (II) from linear measurements. The per cent increase is calculated for the direct determinations (I).

TABLE 2
QUANTITIES OF NITROGEN, RIBOSENUCLEIC ACID (RNA), AND DESOXYRIBOSENUCLEIC ACID (DNA) AND PER CENT INCREASE FOR THE AVERAGE OF FIVE SOMITE STAGES CALCULATED FROM THE DATA IN TABLE 9

Somite stages	Averages (microgms.)			Percentage increase		
	N ₂ /Somite	RNA/Somite	DNA/Somite	N ₂	RNA	DNA
9-15	.050	.041	.021			
16-20	.058	.042	.029	16	2	38
21-25	.098	.090	.053	69	114	83
26-30	.167	.129	.073	71	43	38
31-35	.217	.172	.107	30	33	47
36-40	.284	.213	.123	32	24	15
41-45	.406	.319	.179	43	50	40
46-50	.757	.446	.315	90	40	76

dermatome, myotome and sclerotome, and fusion of the somites takes place subsequently. The cells of the myotome have become spindle-shaped and oriented in an antero-posterior direction. These myoblasts show the first slow contractions by the sixth day. We have found little change in the chemical composition of the muscle tissue in the time from the sixth to the twelfth day. Beginning approximately on the twelfth day of development, however, marked changes take place. One can observe an increase in the ratio of dry weight to wet weight and a more rapid accumulation of proteins than of nucleic acids (TABLE 3). This is in contrast to the decrease of this

ratio in the earlier phase of development and a parallel increase of proteins and nucleic acids. During this phase of development, it seemed of interest to find out to what extent the increase in proteins is due to the development of the specific contractile proteins (actomyosin) of muscle tissue. As a rather specific method for the determination of actomyosin, we made measurements of the viscosity of muscle extracts in the presence and absence of adenosinetriphosphate (ATP), following the method developed by Balenovic and Straub (1942) and Csapo (1950). ATP combines with actomyosin and dissociates the protein molecule into actin and myosin with an accom-

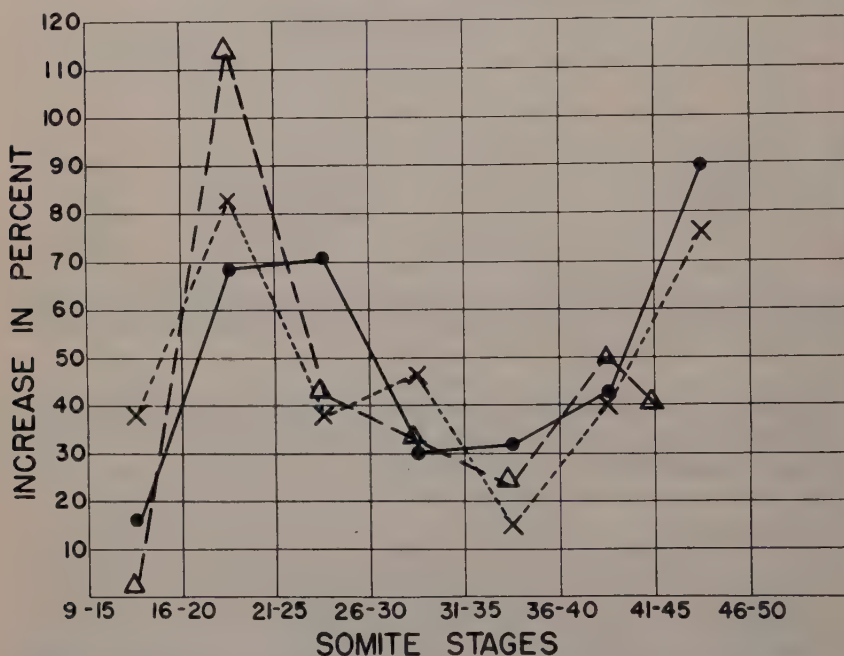


FIGURE 1. Per cent increase in the quantities of nitrogen, ribonucleic acid, and deoxyribonucleic acid for the average of five somite stages. ●—● = nitrogen; ■—■ = ribonucleic acid; and ×—× = deoxyribonucleic acid.

panying sharp drop in the viscosity of the solution of the protein. By standardizing the drop in viscosity with known amounts of actomyosin, the change in viscosity of solutions with the actomyosin concentration of extracts with unknown actomyosin content can be calculated. The determination carried out with this method showed a sharp increase in the actomyosin content beginning as late as the twelfth day of development (FIGURE 2) (Csapo and Herrmann, 1951). We cannot rule out with this method the possibility that small amounts of actin or myosin are present in the extract before this time, or that the initial small quantities of actomyosin are so firmly bound to the cell structure that they cannot be extracted. The contractile proteins derive the energy for contraction from energy rich phosphate esters, adenosinetriphosphate and phosphocreatine. An increase

TABLE 3

QUANTITIES OF DESOXYRIBOSENUCLEIC ACID (DNA), RIBOSENUCLEIC ACID (RNA) AND PROTEIN NITROGEN (PN) IN EMBRYONIC MUSCLE TISSUE OF CHICK FROM THE 9TH TO 20TH DAY OF INCUBATION

(FIGURES INDICATE MICROGRAMS PER 100 MG. WET WEIGHT)

Age	Number of samples	DNA	RNA	PN
9	1	191	388	522
10	2	206	300	749
12	10	191	286	547
13	6	199	328	583
14	16	186	312	712
15	6	214	306	862
16	8	199	299	859
17	10	197	303	1067
18	8	227	335	1167
19	3	178	299	1236
20	6	115	241	6820

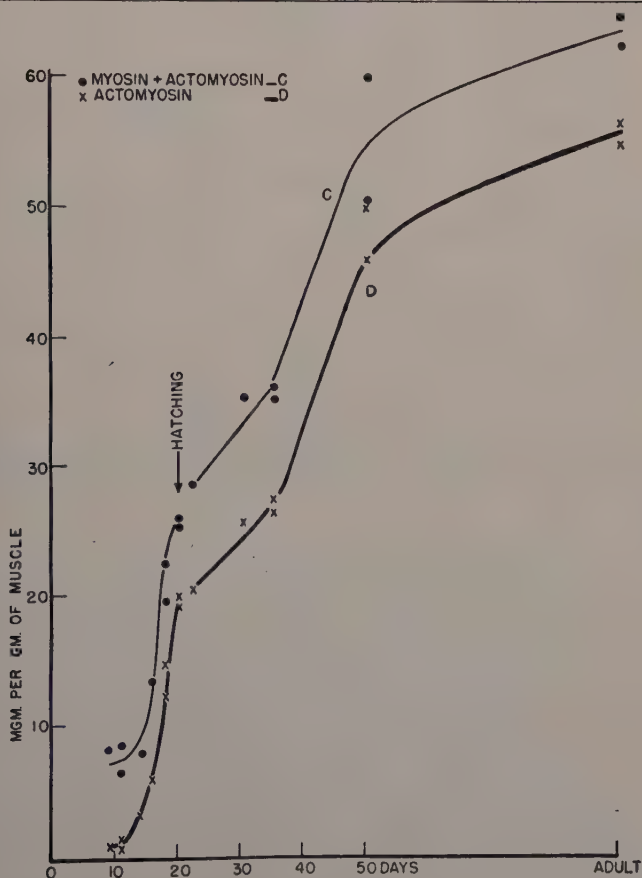


FIGURE 2. Viscosimetric determinations of actomyosin and of actomyosin + myosin in extracts from muscle tissue at different stages of development.

in the concentration of these esters above the level of those found in the six day myoblast does not take place until the 14th to 16th day of development (FIGURE 3) (Herrmann and Cox, 1951). These data show that the production in quantity of the components of the muscle cell, which are responsible for its specific structure and function, does not take place until a late phase in embryonic development.

Discussion

In attempting to give some meaning to this outline of bare facts, the question arises of how to evaluate the observed data. Chemical data on adult tissues can be referred to the dry or wet weight of the tissue or to other

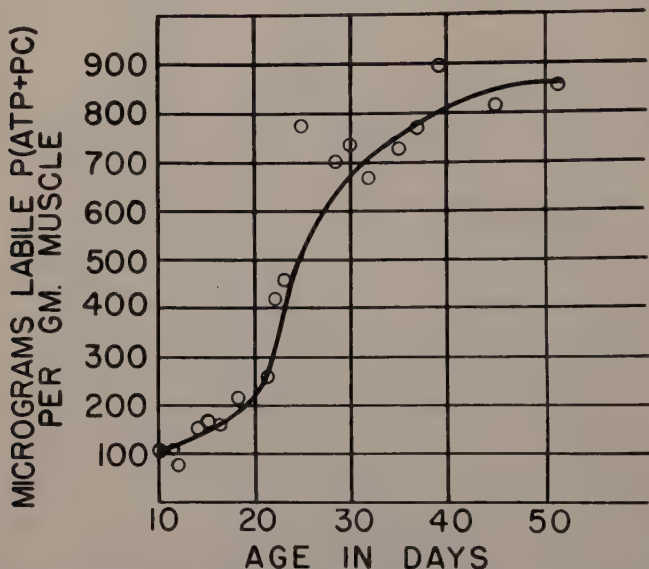


FIGURE 3. Concentrations of labile phosphate expressed as sum of phosphocreatine plus adenosine-triphosphate in muscle tissue of chick embryos from the tenth day of incubation to 52 days of development (31 days after hatching).

arbitrary units. In these cases, the assumption is made that the cell population of the examined tissue remains constant and that the changes in the obtained values correspond to changes in the individual cell. In embryonic tissues which are composed of a population of cells, the number and properties of which change at a shifting rate, such arbitrary units cannot be used as a basis of reference. Only by actually determining the number of cells involved in the measurements and by expressing the observed quantities per cell, a meaningful interpretation of such data can be obtained. Until a short time ago, this was an almost hopeless task because of the tediousness of the determination of cell numbers. With the discovery (Vendrey and Vendrey, 1948; Mirsky and Ris, 1951) of the constancy of the DNA content for different tissues of the same species, this difficulty has been eliminated. The use of the DNA content as a basis of reference in

case of developing tissue has been advocated by Davidson and Leslie (1950). This author and his collaborators obtained data on the later phases of development of several tissues of the chick embryo, including muscle tissue (Davidson and Leslie, 1951). In recalculating our figures per cell of muscle tissues, we used a value of 2.6 micrograms of DNA per cell as given by Davidson and Leslie (1950). Our own determinations in isolated nuclei of embryonic muscle tissue agree well with these figures.

Our recalculated results are listed in TABLE 4. During the development of the somite, from 40 to 84 hours of incubation, the cell number increases in an average somite from 8×10^3 to 121×10^3 . In less than two days, the cell number thus increases by about 15 times. This would correspond to about four divisions of each cell, or to an average intermitotic period of twelve hours. Actually, this period is probably somewhat shorter in the first part and somewhat longer in the latter part of the observed development. The increase in cell number does not account fully for the observed increase in the volume (wet weight) of the somite (26 times, TABLE 1).

TABLE 4
MEASUREMENTS ON SOMITES (40-84 HOURS) CALCULATED PER CELL

<i>Age (hours)</i>	<i>Cell number/ somite $\times 10^3$</i>	<i>Cell mass (mg $\times 10^{-7}$)</i>	<i>PN/cell (mg $\times 10^{-9}$)</i>	<i>RNA/cell (mg $\times 10^{-9}$)</i>
40	8.08		6.19	5.09
48	11.1	3.60	5.22	3.78
53	20.4	2.94	4.81	4.31
60	28.1	3.56	5.94	4.60
66	41.2	3.88	5.27	4.18
70	47.4	6.32	5.99	4.49
78	68.9	9.60	5.90	4.62
84	121.0	8.00	6.25	3.68

Therefore, we assume that at least a greater part of the difference in the increases in cell number and somite volume is made up by an increase in cell volume. Since the protein content per cell during this period remains within the range of 5 to 6×10^{-6} microgram, the increase in the cell volume must be due to an increase in the water content of the cell. The ratio of $26/15$ would give a dilution of the cell content during this period of development by about 1.7. In comparing the values of PN, RNA and DNA during somite development, it will be noted that the values per cell remain within a narrow range if we neglect smaller deviations, the significance of which we cannot evaluate at the present time. The changes in the rate of accumulation of these substances in the developing somite, which are demonstrated in FIGURE 1, therefore can not be due to variations in the accumulation of these substances in the cell. Rather, they must mean that the proliferation of cells itself occurs in spurts. Actually, it seems rather remarkable that such wide variations in the rate of cell proliferation can occur without corresponding changes of the protein and nucleic acid content per cell. The existence of such spurts of proliferation has been claimed by several authors, and has been emphasized in the work of Schmalhausen (1926). As an ex-

planation, it has been proposed that the periods of proliferation and differentiation alternate. These statements have been based on rather insufficient analyses, however, and recent work of Hamburger (1948) shows that early differentiation in the spinal cord is actually accompanied by an increase in proliferative activity.

Following the somite development, we find a period of about five to six days in which very little change can be noticed in the observed magnitudes. Some new trends, which have been indicated previously, become recognizable only after the eleventh day of development. While the RNA and DNA content per cell still remain within the narrow range, the protein content per cell begins to rise sharply after this period. Up to the eighteenth day, the increase in protein content is not followed by a significant increase in cell volume (wet weight). That must mean that water content of the muscle cell is being substituted by protein, with a corresponding increase of

TABLE 5
MEASUREMENTS ON EMBRYONIC MUSCLE TISSUE (9-20 DAYS)
CALCULATED PER CELL

Age (days)	Cell number/100 mg wet weight $\times 10^6$	Cell mass (mg $\times 10^{-7}$)	PN/cell (mg $\times 10^{-9}$)	RNA/cell (mg $\times 10^{-9}$)
9	73.5	13.6	7.10	5.28
10	79.2	12.6	9.45	3.79
12	73.5	13.6	7.45	3.89
13	76.6	13.1	7.60	4.28
14	71.5	14.0	9.95	4.37
15	82.4	12.2	10.50	3.72
16	76.6	13.1	11.20	3.90
17	75.9	13.2	14.10	4.00
18	87.3	11.5	13.40	3.83
19	68.5	14.6	18.10	4.36
20	44.2	22.6	25.7	5.45

dry matter per cell. Later on, the cell volume (wet weight) seems to increase as quickly as the protein content. This observation leads again to the distinction of two separate mechanisms, protein production with and without increase in cell size. Assuming that cell proliferation in muscle tissue has become negligible after the twelfth day of incubation, the entire increase in muscle mass is probably due to protein production by the individual cell. About one-fourth to one-third of these newly formed proteins is the specific contractile protein, actomyosin.

The development of the muscle cell resembles, in some aspects, developmental processes in the nervous system. Levi-Montalcini and Levi (1943) have distinguished three phases in the development of the spinal ganglia. First, a segregation of two types of cells occurs which is accompanied by a relatively high mitotic activity, corresponding to the segregation of cell types in the somite. A period of little change in cell development then follows, both in the spinal ganglia and in muscle. In a terminal phase, quantity production of specific cytoplasmic structures takes place. In the

nervous tissue, we have the formation of neurons and, in muscle, the formation of contractile fibers.

What the factors are which regulate proliferation and differentiation is indeed a problem of considerable difficulty. To what extent retardation of proliferation, or, to what extent more specific mechanisms are responsible for cytoplasmic differentiation is still a wide open question. Stockard (1921) and, in more recent times, Bodenstein (1949) and Vogt (1947), have attempted to bring evidence in support of the first alternative. Landauer's work (1948) on the effect of insulin on differentiation of the skeleton at various phases of development of this tissue supports the second alternative. It is hoped that the further analysis of the chemical differences of the three phases of muscle development will provide useful material for the solution of this problem.

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DEVELOPMENT OF THE DIGESTIVE TUBE AND ITS DERIVATIVES

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The chick embryo is the type used almost universally to illustrate the course of morphogenesis in higher vertebrates. It is scarcely necessary therefore to recall that the digestive tube and the glands and diverticula associated with it originate, during the first day of incubation, from a flat tissue sheet, the so-called splanchnopleure, which is composed of two well-defined layers: a lower flat epithelium, the entoderm, to the upper surface of which is closely applied a mesoblastic mesenchymal layer. The mechanics of folding are such that, except in the anteriormost region of the embryo, the mesenchymal component surrounding the gut tube originates from the lateral portion of the embryonic mesoderm, the so-called splanchnic mesoderm of the lateral plate. The epithelial and glandular elements of the digestive and respiratory tracts derive of course from the entodermal epithelium. The muscular, vascular, connective and serous elements derive from the splanchnic mesoderm. The various organs of these systems are formed either by enlargement or by elongation of the original tube, or by localized outgrowths of its wall.

The origin of the two elements which compose the splanchnopleure is by no means as well understood as it seemed to be twenty years ago. At the time an egg is laid, or very shortly thereafter, in the first phase of gastrulation, a lower layer or hypoblast is cut off from the blastoderm. Such a lower epithelial layer is found at all subsequent blastoderm stages and ordinarily has been believed to be the entoderm itself. Experimental work (*cf.* Hunt, 1937b) has suggested the possibility that the early hypoblast, during the activity of the primitive streak which marks the second phase of gastrulation, is reinforced by cells invaginated from the surface layer, through the primitive streak, and, therefore, that the hypoblast is not the exact equivalent of the entoderm present after gastrulation is complete. There is no suggestion in the literature that the primitive streak gives a mass contribution to the hypoblast; rather that individual streak cells enter the lower layer without disturbing its continuity. Hunt (*op. cit.*) and Spratt (1946) have described such cells as occurring in preparations growing *in vitro*. These cells, if this version is correct, are of great importance developmentally, as will be pointed out shortly. As for the origin of the splanchnic mesoderm, investigators agree that it is invaginated from the upper layers through the primitive streak; Pasteels (1936) has marked the region of the early streak that apparently provides lateral plate mesoderm. Whatever the ultimate verdict will be on the question of whether entodermal cells actually originate in the primitive streak, there is no doubt that, once the streak is active, the forming and spreading mesoderm adheres very closely to the underlying hypoblast. Thus, there is justification for considering the lower mesoderm and the entoderm as a unit, as we do in the present discussion.

The senior Wilhelm His (1874, page 80) was the first author, to my knowledge, to publish a diagram showing the projection of organs of the digestive and respiratory complex back onto the flat unfolded embryonic splanchnopleure, as in the head-process blastoderm. If we ignore some confusion about the origin of the thyroid, which His evidently believed to derive from two lateral pouches instead of a median ventral one, we find that the diagram reproduced in FIGURE 1 applies adequately in the present connection, representing what we call prospective areas, that is to say, the cellular regions, antedating any visible rudiment, that will actually enter the composition of the rudiment and the organ in question. In the flat sheet, antero-posterior order is unaltered, whereas dorsal structures lie medially, and ventral organs are double and lateral. It must be added that nobody, to my knowledge, has ever marked and traced any of these splanchnopleural areas. The experiment remains as in 1874, imaginary.

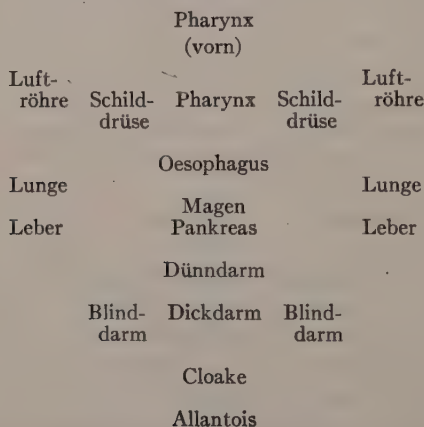


FIGURE 1. Prospective areas in the splanchnopleure of the chick. Diagram from His (1874), p. 80.

If we wish to attempt tracing these prospective areas further back in development, to primitive streak stages, some assumptions must be made. Marking experiments tracing the formation of the mesodermal component of the splanchnopleure are available, though not as detailed as one might desire. We know almost nothing about independent movements of the hypoblast or entoderm in this period. It has been assumed, in order to make the diagrams in FIGURE 2, that lateral mesoderm and entoderm behave as a unit in primitive streak stages as they do later. In this figure, Diagrams 1 and 4 show respectively primitive streak and head-process splanchnopleure under this assumption, whereas 6 shows the outcome at a three-day stage, when the foregut and hindgut are already closed and the various subdivisions clearly visible. It has also been assumed that in the period between 1 and 4, the mesoderm anterior to Hensen's Node is already in its definitive place, and takes part in formation of the embryonic axis by movements corresponding to those that have been delineated by Wetzel (1929) and Spratt (1947) for the ectoderm.

The classic problems are as follows: Given, that we know the prospective areas in an apparently structureless sheet, do these areas differ from one another at this early time? When do differences between them arise? Is the agency intrinsic or external to the area in question?

In attempting answers, embryologists have done considerable cutting and dissecting of the blastoderm, followed by transplantation of pieces to suitable graft sites, such as the chorio-allantoic membrane. Diagrams 2 and 5 of

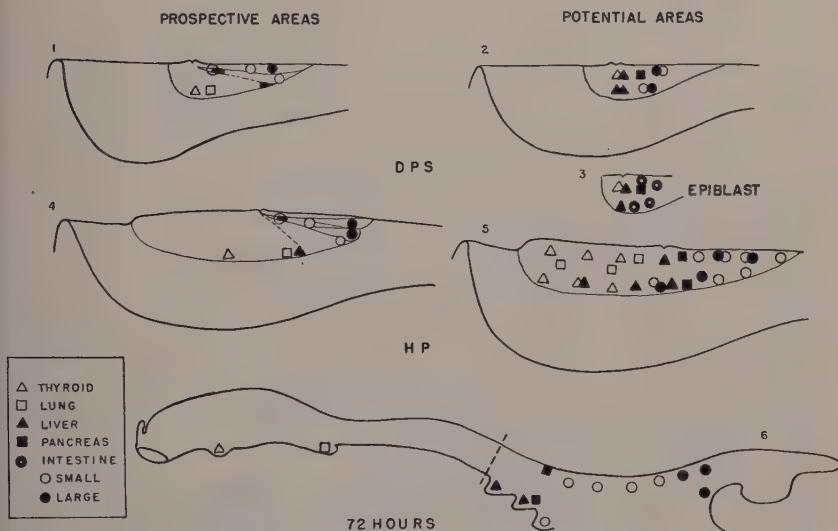


FIGURE 2. Diagrams showing the development of prospective as compared with potential areas of the splanchnopleure:

1. Prospective areas for thyroid, lung, liver, pancreas (dorsal) and intestine in the definitive streak stage. The blastoderm is represented as folded longitudinally so that median structures appear in their future dorsal position. Data from marking experiments.
2. Potential areas for the above organs in the definitive primitive streak stage: from results of differentiation in chorioallantoic grafts. All three germ layers included in transplants.
3. Potential areas as in Diagram 2 when hypoblast was not included in the grafts.
4. Prospective areas as in Diagram 1, for the late head-process stage. Foregut material now mostly anterior to the primitive atreum. It has been assumed that splanchnopleure areas have not moved relative to the overlying ectoderm in the interval between Diagrams 1 and 4.
5. Potential areas in the head-process stage. The antero-posterior order approximates that of prospective areas; the medio-lateral distribution does not.
6. Diagram of the nearly closed gut tube of the three-day chick. At this stage, most organ rudiments are visible, and potencies correspond with prospective significance.

FIGURE 2 summarize results of actual differentiation of the major splanchnopleure derivatives; these have been compiled from data of Willier and Rawles (1931a); Hunt (1931); Rudnick (1932, 1933); and Rawles (1936). It is apparent, at once, by comparing these with Diagrams 1 and 4, that the regions from which a tissue or organ may differentiate, *i.e.*, the potential area, does not exactly coincide with the corresponding prospective area. The correspondence is rough, not precise. As the embryonic axis is established and the gut tube closed, the correspondence does become precise. Diagram 6 does equally well in showing prospective and potential areas.

In connection with the problem proposed earlier, whether the entodermal layer receives a contribution from the overlying primitive streak, Diagram 3 indicates the results if the hypoblast is entirely omitted from transplants

(Hunt, 1937a) in the primitive streak stage. It will be noted, by comparison with Diagram 2 above, that the distribution of potencies is almost unaffected. It is necessary to think that some potential material, at least, is invaginated by the streak. This might include the entodermal cells themselves, or possibly mesodermal material capable of inducing labile cells to form the entodermal structures in question.

Organization in the Potential Areas. If we take into consideration not only the spatial distribution of potencies in blastoderm stages, but the quality of differentiation of the various organs under conditions of transplantation, we can recognize at least two categories of organ. The first group is represented by thyroid, liver and intestine. These structures differentiate in grafts with considerable regularity and remarkable specificity. Thyroid masses in grafts are histologically indistinguishable from the normal tissue, except that their differentiation is evidently capable of some sporadic acceleration (Rudnick, 1932). Morphologically, graft thyroids are very irregular and do not at all resemble the normal organ. This is also true of liver (Willier and Rawles, 1931b; R. H. Sandstrom, 1934). Intestine, both small and large (Rawles, 1936; Rudnick and Rawles, 1937) forms with remarkable specificity, with all tunics, and usually in a coelomic space. If the assumptions made in FIGURE 2 are at all justified, many of the reported cases of these very specific structures, in grafts, have differentiated from areas which would have formed something quite different if left undisturbed. Thus, it may be said that the potential areas of thyroid and liver, at least, encroach considerably on neighbouring areas.

For other parts of the digestive system, notably foregut tube and respiratory tract, the situation is different. Grafts of pieces of blastoderms do indeed contain many tubes which bear distorted resemblance to foregut and respiratory tubes; but the differentiation is without real precision. The epithelial component grades from one type to another in very short space. The mesodermal tunics may or may not be present and, if present, may or may not be specific enough for diagnosis. In short, tubes, apparently of splanchnopleural origin, seem to have proliferated through these grafts without regional restraint or spatial organisation. This lack of order is less surprising if we note that, even after regional anlagen of the foregut tube (*e.g.* the proventriculus) are morphologically distinct, if grafted, they may differentiate rather imprecisely, especially in epithelial type (Rudnick and Rawles, 1937).

The lung, as an example of this category of organ, will not differentiate fully histologically or morphologically if transplanted before the rudimentary bud actually appears on the third day of incubation (Rudnick, 1933); and even then, the number and pattern of secondary and tertiary branches is extremely variable.

Role of the Mesoderm. The question of the nature of the potencies that have been demonstrated, whether intrinsic in the layer that actually performs the differentiation or imposed from outside, cannot be answered directly from grafts, since in all cases at least two of the three germ layers were included in transplants. Some indirect evidence exists:

Thyroid and liver are both cases where the differentiation involved is primarily epithelial, normally, of course, entodermal. In grafts, a strong tendency of both these tissues to be associated with heart was reported early (Willier and Rawles, 1931b) and the possibility was suggested that heart mesoderm may induce these two glandular potencies. It might be preferable not to specify heart mesoderm alone. Cases have been reported where liver or thyroid has developed in the absence of heart, even from pieces including only prospective foregut roof (Rudnick, 1935) from which heart was already excluded. In analysing this situation, it was noted that such thyroid and liver was not found to differentiate from gut roof pieces after the beginning of somite formation, unless the differentiation of the dorsal axis had been artificially retarded with respect to what was happening ventrally (closure of the foregut and heart formation). Thus, the loss of thyroid and liver potencies from the roof of the foregut is not something that occurs merely with the passage of time, nor is it correlated with heart formation, but rather with morphogenesis in the layers above the roof.

In the case of the lung bud, it has been shown that the secondary and further branching of the entodermal tube, together with characteristic differentiation of the lung parenchyma, will not occur if the rudiment is divested of its outer mesenchymal coat. FIGURE 3 summarizes the morphogenetic situation in the lung. The mesoderm surrounding the bud forms rather early, it will be recalled, as an appreciable cushion into which the bud grows and branches. No damage is done to the epithelium by removal of this coat, since the outer mesoderm splits off freely, leaving an inner thin mesenchymal layer protecting the epithelial bud. This experiment suggests at least a mechanical role of mesoderm in this particular organ formation; possibly more.

Cytological Stability: The Liver. During the first four days of normal development, an entodermal cell destined to become liver parenchyma undergoes a series of alterations: first in common with the rest of the foregut; and later more specific, as the diverticula bud and branch in the region of the vitelline veins. Dalton (1937) has figured the cytoplasmic changes thoroughly. There are also nuclear changes, involving accumulation of acid phosphatase (Moog, 1944).

Lynch (1921) has given us a good survey of the behavior of explanted hepatic cells during development in the chick. Using short-time cultures in fluid medium, she found that even the five-day liver rudiment can give rise to membranes of characteristic hepatic cells, in addition to endothelial and serosal elements. Others (see Levi, 1922, 1934, *etc.*) have verified the fact that as soon as there is a rudiment capable of isolation, hepatic cells will migrate freely from it *in vitro*. Strains of these cells (Bisceglie, 1931; Doljanski, 1931) will retain their specificity for months *in vitro*, after repeated mitoses and transfers. The cytological characters that appear so stable are evidently the profusion of mitochondria and possibly a peripheral arrangement of sudanophile droplets, together with the characteristic epithelial tendency to form membranes and to spread on surfaces. It is not clear whether the nuclear specialization so striking in the rudiment and in

grafted tissue, is retained in these migrating cells. The hepatic cells apparently do not retain their specificity long when they wander off by themselves. The persistent differentiation seems to depend on the existence of a large number of cells of the same kind in contact. Mesodermal elements are quickly eliminated by their differential behavior *in vitro*.

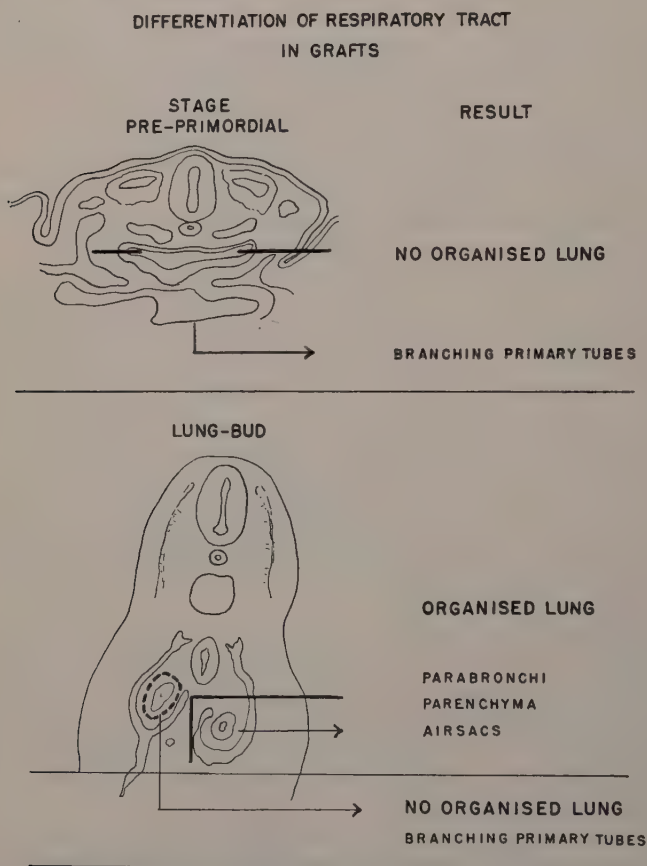


FIGURE 3. Diagrams showing degree of differentiation of the lung in chorioallantoic grafts: (a) when foregut floor is transplanted before the appearance of lung buds; and (b) when lung bud is transplanted with or without mesodermal outer coat.

Several workers have observed that liver rudiments older than 11-12 days' incubation yield very poor epithelial emigration when explanted. Nordmann (1929) has ascertained a gradual slowing of emigration rate during the second half of incubation, which he has correlated with progressive accumulation of lipid within cells. Indeed, the curve published by Dumm and Levy (1949) shows just such steady acceleration in lipid growth of the liver in this period. Other types of cell *in vitro*, however, may be uninhibited in motility by tremendous lipid loads, and some additional

factors must be invoked. The same inhibition, amounting to actual degeneration after the 11–12 day stage, has been noted in grafts of liver (Sandstrom, 1934), where cellular motility is not important.

If we ask about the origin of this cytological specificity of the early liver rudiment, combined as it is with quite free cellular mobility in the period before 11 days' incubation, an easy answer is not forthcoming. Levi (1925) has pointed out that early blastodermal tissues, when explanted, yield a characteristic sort of epithelial cell. I have examined a large number of explants of the hypoblast of primitive streak stages, and can confirm that the basic epithelial tendency to form membranes is well represented there. Explants of the primitive streak itself will form membranes, but, in addition, other independent cell types. So far, I have never found any cell that resembles a liver cell in either hypoblast or primitive streak explants, even after a suitable time has passed *in vitro*.

Thus, in the primitive streak stage, when limited areas of the blastoderm, excised and transplanted to the chorio-allantois, will, in an appreciable percentage of cases, yield masses of liver cells (among other kinds), mobilized epithelial cells evidently do no more than form a very embryonic sort of epithelium. Later, after a rudiment is formed, the liver epithelium can persist or progress in differentiation under the most disruptive circumstances. The events occurring between these two periods seem deserving of close investigation.

Summary

This brief and selective survey has attempted to illustrate for the gut derivatives the following points: (a) the topographical relation of potencies to prospective areas, in so far as they have been analysed; (b) the problems of the relationship of mesoderm to entoderm (these problems still remain in a most unresolved state); and (c) the problem of cytological specificity in one case, that of the liver, where a considerable body of experimental information has been accumulated, and where we may hope for further elucidation as cytochemistry develops. It is obvious that the analysis of these problems has not been carried to the limits of modern techniques and it is also obvious that we are still ignorant of many of the subtler cellular events of normal development; for example, witness the confusion as to the origin of the entoderm—a confusion, it must be said, that is not confined to the situation in the chick embryo.

The splanchnopleure derivatives possibly have not been the favorite of embryologists in the past. As developmental metabolism studies become more and more possible, our knowledge of this group of systems will need to be expanded. We have tried to indicate in this discussion points of entry on the cellular, tissue, and organ levels for future exploitation.

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DEVELOPMENT OF THE NERVOUS SYSTEM

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The nervous system of the chick embryo has become increasingly important for neuro-embryological studies. It is more highly organized than that of the Amphibia which has been the classical object for the pioneer investigations in this field. At the same time, it is equally accessible to experimental analysis, which gives it a definite advantage over that of the mammals. The rapid development of the chick embryo and the excellent results which one obtains with silver impregnation techniques are additional assets. Since many centers are well circumscribed, and not excessively large or complex, simple quantitative methods can be applied, such as cell counts and area measurements.

In a previous symposium, D. Rudnick (1948) has given a lucid account of the origin of the medullary plate. Therefore, we shall pass over this first chapter of neurogenesis and take the neural tube of the two-day embryo (15–25 somites) as the starting point of our discussion. The cells of the neural epithelium are closely packed and interdigitated, and they do not show any visible differentiation. The main parts of the brain, however, are already blocked out and set off from the spinal cord.

The Organization of the Neural Tube

The spinal cord of this stage has been the object of a number of experiments which have revealed a considerable degree of organization in this structure. A rather rigidly determined pattern of regional differences was found in heterotopic transplantation experiments in which the cervical, brachial and thoracic sectors of the cord were substituted for each other (B. Wenger, 1951; Shieh, 1951), following the classical procedure which Detwiler (1923, 1936) inaugurated in Amphibia. The cell types and cellular arrangements characteristic of each level developed in the normal fashion, and not according to the site of implantation. For instance, the thoracic or cervical segments, which were implanted in the brachial level, innervated the wings and their nerves formed plexuses approaching the normal brachial nerves, but the impact of the peripheral overloading failed to induce a lateral motor column in the cervical and thoracic cord segments. On the other hand, the brachial cord retained the region-specific lateral motor column when transplanted to other levels. This example illustrates the high degree of inherent regional organization. A notable exception which was found in the motor system of the cervical cord will be discussed below. The transplantation of isolated parts of the cord, taken from different levels and implanted in the lumbosacral region between the somites and the hind limb bud of the host, gave essentially the same results (Bueker, 1943, 1945). The typical configuration of the nerve centers in the transverse plane is likewise fixed at these early stages (E. Wenger, 1950). In contrast to the situation in Amphibia, no regeneration or regulation occurs

in the early neural tube. The extirpation of lateral and dorsal halves of the brachial level in embryos of 15-25 somites resulted in a wide range of deficiencies in the mantle which could be correlated with corresponding deficiencies in the ependymal layer. It was found that a mosaic of at least six separate units exists in the early neural epithelium, along the dorso-ventral axis. Each sector gives rise to only those specific types of neurons which originate from it in normal development. No interactions that could be called "embryonic inductions" were observed between the different parts of the neural epithelium.

These early established patterns represent a general framework of organization. Many details within this framework are not yet rigidly determined, and very complex interactions between the different elements of the central nervous system and between nervous and non-nervous elements operate during the following period of progressive differentiation to establish the final configuration. In particular, the quantitative aspects of neurogenesis are subject to control by intrinsic and extrinsic agents.

The Spinal Cord at Eight Days

The nervous system of the eight- to ten-day embryo shows a remarkable increase in visible complexity which approaches the organization of the adult system (FIGURES 1, 2). The proliferative phase is nearly terminated, which means that the spinal cord of the eight-day embryo contains practically all potential neurons which will be present in the adult cord. The different cell groups have migrated to their destinations in the mantle and have formed the main centers. The size differences of the different types of neurons are apparent. The peripheral nerves have grown out and established provisional connections with non-nervous structures, and the intracentral fiber tracts either have formed their specific synaptic connections or are in the process of doing so. Obviously, the experimental analysis of the mechanisms of neurogenesis must concentrate on this period between the second and ninth to tenth days of incubation.

The analysis is facilitated by the fact that the three main aspects of progressive development, namely, proliferation, differentiation of neuroblasts, and cell growth, are quite well separated in space and time, and a fourth component, morphogenesis, does not enter into the picture, at least not in the spinal cord, which remains a simple tube. On the other hand, the situation is complicated by the fact that the unit of nerve tissue organization is not the nerve cell but the nerve center, although differentiation, proliferation and growth are functions of individual cells. Hence, the investigator is constantly confronted with events which happen on two levels, the cellular and a supercellular level. Limitation of space permits the discussion of only a few general correlations and concepts which have emerged from descriptive and experimental studies.

Mitotic Activity

It is logical to begin with the first component, proliferation. It is well known that all mitoses are located in the lining of the central canal. In an

extensive study of proliferation between the third and eighth days of incubation (Hamburger, 1948), mitoses were counted separately in the dorsal and ventral halves of the spinal cord. It was found that the distribution

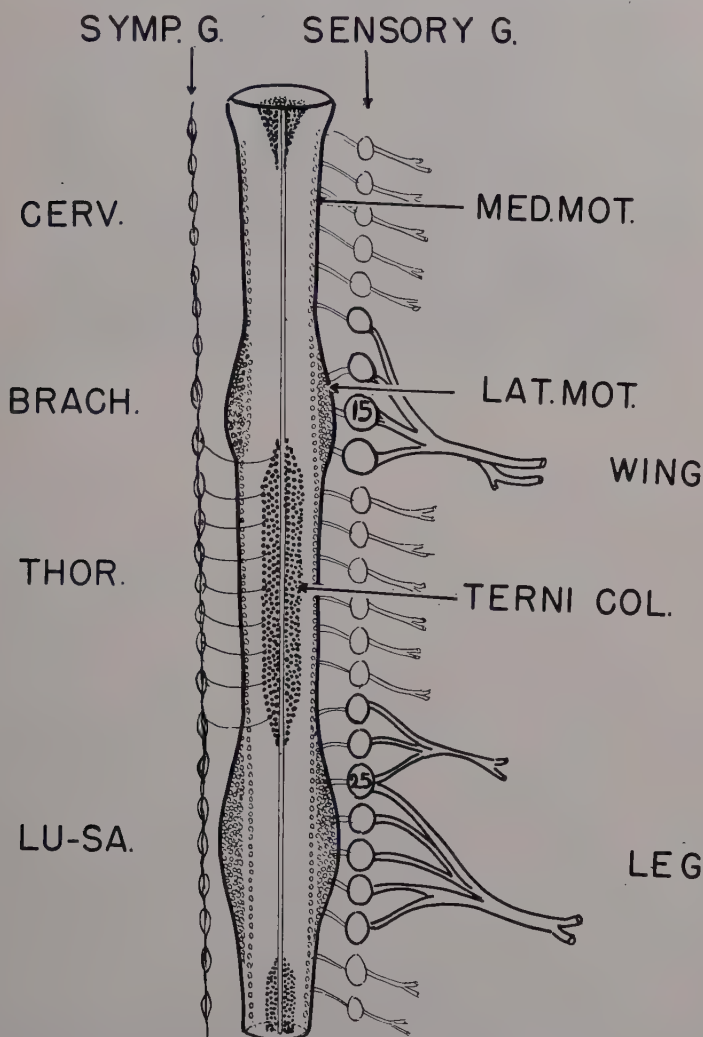


FIGURE 1. Diagram of spinal cord of eight-day embryo. Brach., brachial level; Cerv., cervical level; Lat. Mot., lateral motor column; Lu-Sa., lumbo-sacral level; Med. Mot., median motor column; Terni Col., preganglionic column of Terni; Thor., thoracic level.

curves in the alar and basal plate, respectively, represent very different and, in some respects, contrasting, features (FIGURE 3). The peak of mitotic activity in the basal plate is at three days, and that of the alar plate is at six to seven days. Between three and six days, the mitotic activity rises in the alar plate and declines in the basal plate; between six and nine

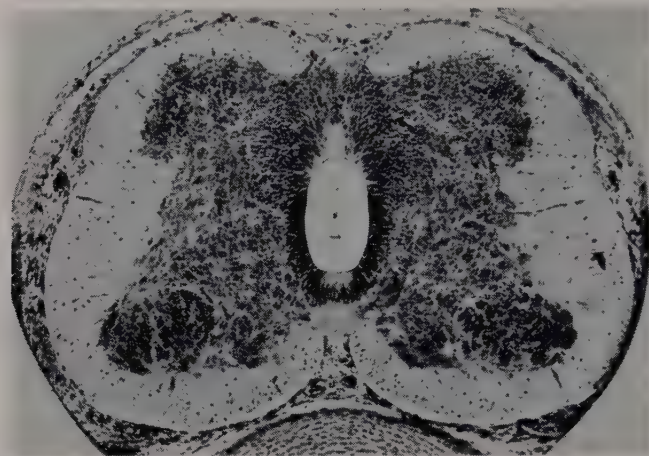


FIGURE 2. Cross section through brachial level of spinal cord of eight-day embryo.

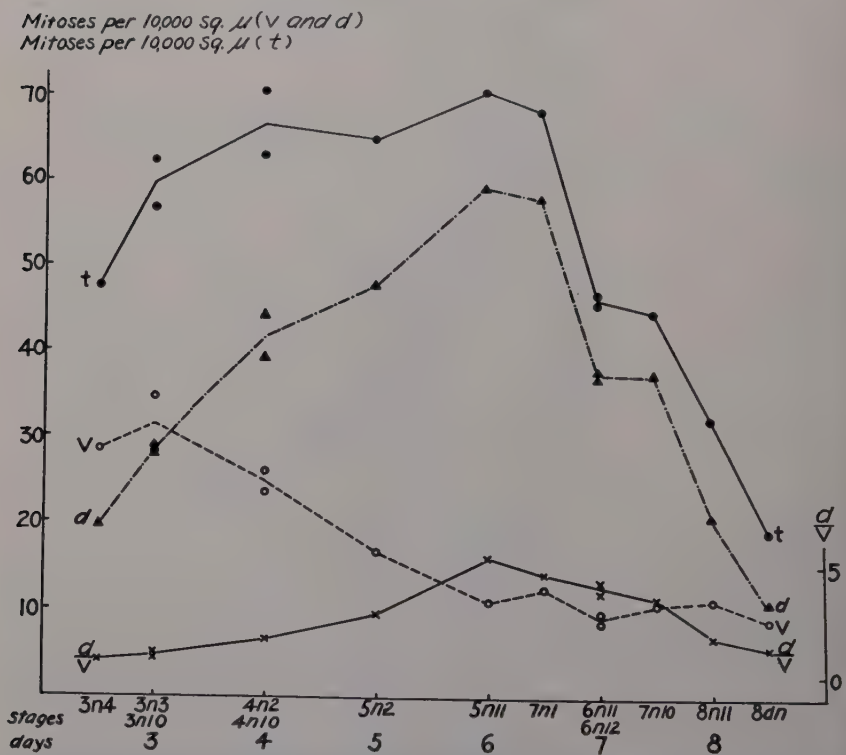


FIGURE 3. Time pattern of mitotic activity in spinal cord of chick embryo (averages for segments 10-20). *Abscissa*, stages of spinal cord differentiation and estimated chronological age; *ordinate* (left), average number of mitoses per unit area of lining of central canal; *ordinate* (right), ratio of mitoses in dorsal half (d) to mitoses in ventral half (v). (From Hamburger, 1948, *J. Comp. Neurol.*, Vol. 88, Figure 5.)

days, proliferation in the alar plate declines sharply, but in the basal plate it retains a uniform, though low, average. The absolute figures are consistently higher in the alar plate than in the basal plate. For instance, the spinal cord of a six-day embryo contains approximately 20,000 mitotic figures, of which only 20 per cent are located in the basal plate. Generally speaking, the mitotic activity in the spinal cord is a patterned process, and proliferations in the basal and alar plates are independent of each other, both quantitatively and with respect to time patterns. This implies that agents which stimulate or control the proliferative activity of the neural epithelial cells must be distributed in a rather complex fashion along the dorso-ventral axis of the neural tube (for further discussion see Hamburger, 1948).

The time pattern of mitotic activity can be correlated rather closely with the subsequent histogenetic events. For instance, the motor columns make their appearance three to four days in advance of the dorsal sensory columns and this time sequence is reflected in the mitotic pattern. The peaks of mitotic activity in the dorsal and ventral halves are approximately $3\frac{1}{2}$ days apart.

Origin of Regional Differences in the Motor System

In another respect, no such correlation was found where it might have been anticipated. Since the motor columns are much larger in the limb levels than in other levels, one might have expected peaks of mitotic activity in these regions. The proliferative activity, however, was found to be rather uniform along the entire cephalo-caudal axis of the spinal cord, in all stages. This seemingly paradoxical situation found its explanation when the origin of regional differences in the motor system (FIGURE 4) was studied in detail (Levi-Montalcini, 1950). It was found that the motor system in its earliest stages (3–4½ days) is represented by a ventro-lateral column which is of uniform size throughout the cord (FIGURE 4a). The regional differences are brought about in the following way: in the cervical level, a considerable number of neuroblasts undergo cytolysis and disappear; and in the thoracic level, a large group of neuroblasts migrate from the ventro-lateral column in a medio-dorsal direction (FIGURE 6, P). These cells settle near the central canal where they form the preganglionic column of Terni. In the limb levels, the column is not depleted, either by migration or by cytolysis (FIGURE 4, B, C). In other words, it is not the differential mitotic activity, but local depletions by cellular breakdown and migration, which are instrumental in the final regional specifications. These findings caution us against an all too common mistake: to attribute all quantitative differences, indiscriminately, to differences in proliferation. These differences bring cytolysis and migration into focus as instruments of pattern formation. The neurologist will be interested in another point, namely, that the visceral and somatic motor systems have a common morphological origin, although it is not contended that the early motor column is structurally homogeneous. It is conceivable that the somatic and visceral elements are already determined at that early stage.

The disappearance of part of the cervical motor system is rather puzzling, and one wonders why a similar breakdown does not occur in other parts of the column. A study of neuroblasts before their disappearance showed that they were connected by rami communicantes with a temporary sympathetic chain, and the idea suggested itself that we may be dealing with an

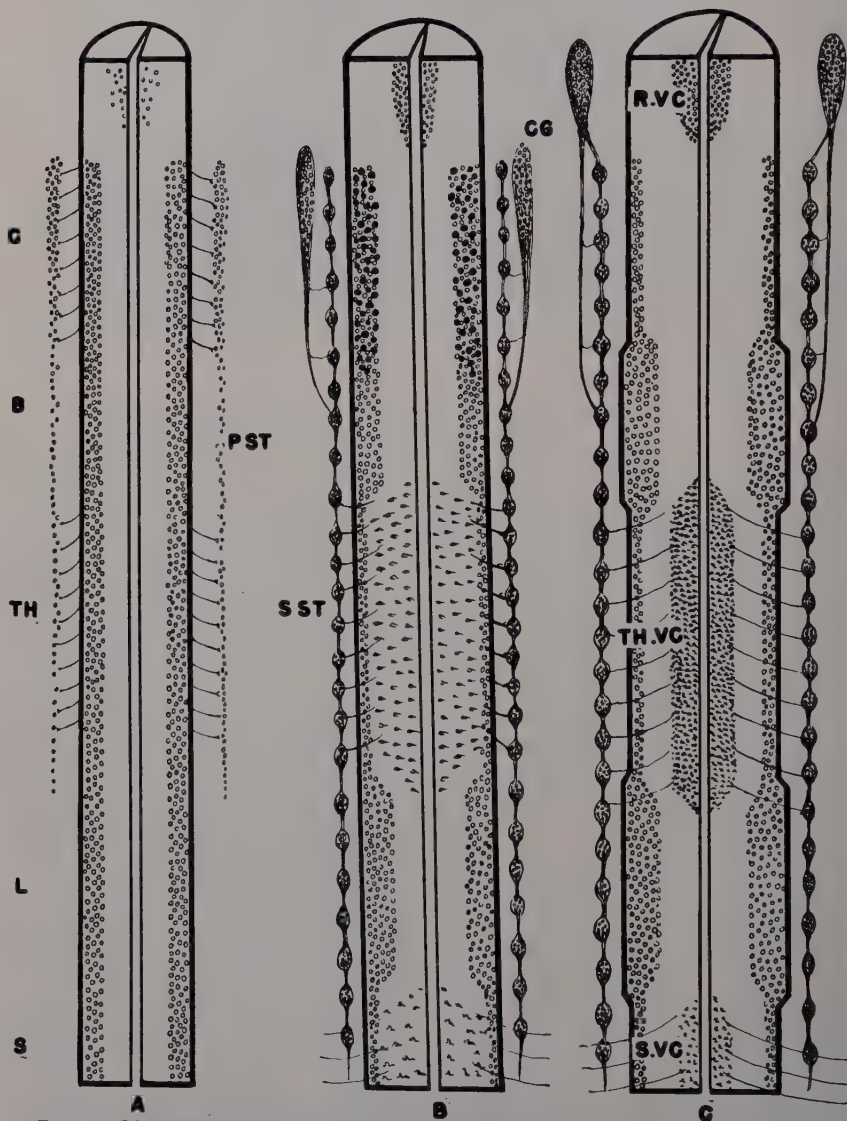


FIGURE 4. Diagrammatic frontal sections of the spinal cord of chick embryos, showing the emergence of regional differences in the motor system from a morphologically uniform system. A, 4 days; B, 5 days; C, 8 days. B, brachial level; C, cervical level; CG, cervical ganglion; L, lumbar level; PST, primary sympathetic trunk; R.V.C, rhombencephalic visceral center; S, sacral level; SST, secondary sympathetic trunk; S.V.C, sacral visceral center; TH, thoracic level; TH.V.C, thoracic visceral center (nucleus of Terni). (From Hamburger and Levi-Montalcini, 1950, *Genetic Neurology* (ed. P. Weiss).)

abortive and short-lived cervical preganglionic system. It was then thought that if the cervical and thoracic levels are akin in their potentiality to form preganglionic systems, it might be possible to activate this inherent capacity of the cervical cord by transplanting it in the place of the thoracic cord. In this position, optimal conditions would exist for the actual differentiation of a preganglionic system, mainly because an opportunity would be given for synaptic connections with the normal paravertebral sympathetic chain. This experiment (FIGURE 5) was performed by Shieh (1951). A migration

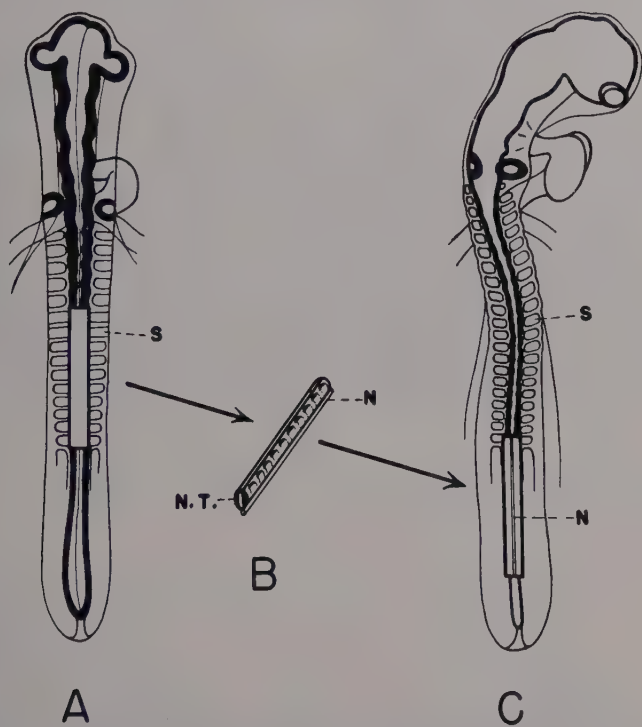


FIGURE 5. Transplantation of cervical spinal cord to the thoracic level. A, donor with cervical spinal segments including the notochord removed; B, transplant; C, host with implantation site; N, notochord; NT, neural tube; S, somites. (From Shieh, 1951, *J. Exp. Zool.*, Vol. 117, Figure 1.)

of motor cells was actually observed in the posterior segments of the transplanted cervical cord (FIGURES 6, 7). As a result, preganglionic cell groups formed which resembled closely the nucleus of Terni, both in position and in mode of origin. In a few instances, neurites of these cells were traced to rami communicantes. This experiment confirms the assumption of an abortive preganglionic system in the cervical cord. It reveals, at the same time, an exception (so far the only one) to the statement made above, that regional differences in the cord are rather rigidly fixed in early stages. Since we do not know which particular local conditions in the thoracic level call forth the neoformation of preganglionic cells, we prefer not to label it as an "embryonic induction."

Cytolysis

Differential cytolysis plays a role in the creation of quantitative differences of other parts of the nervous system. For instance, it was found that the size differences of spinal ganglia are due, in part, to the operation of this mechanism (Hamburger and Levi-Montalcini, 1949). During the

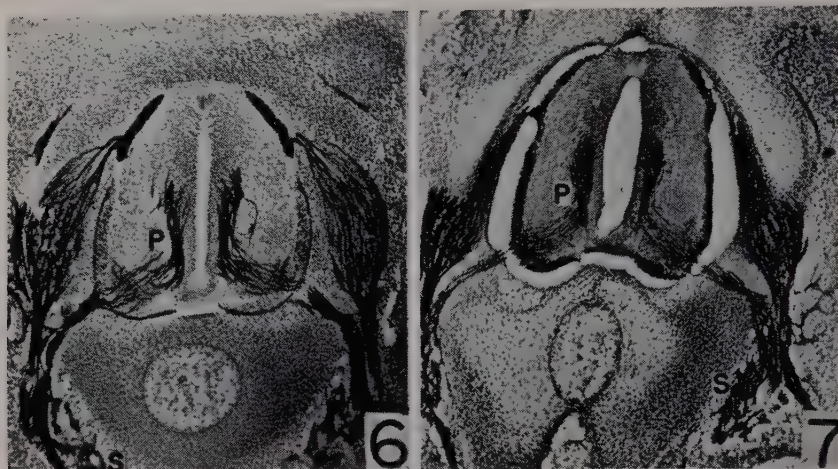


FIGURE 6. Cross section through the median part of the normal thoracic spinal cord of 6½-day embryo. P, preganglionic nucleus of Terni; S, sympathetic ganglion. (From Shieh, 1951, *J. Exp. Zool.* VI17, Figure 12.)

FIGURE 7. Cross section through the middle part of a cervical segment transplanted to the thoracic level. Embryo fixed at 6½ days. P, preganglionic column of Terni; S, sympathetic ganglion. (From Shieh, 1951, *J. Exp. Zool.* Vol. 117, Figure 13.)

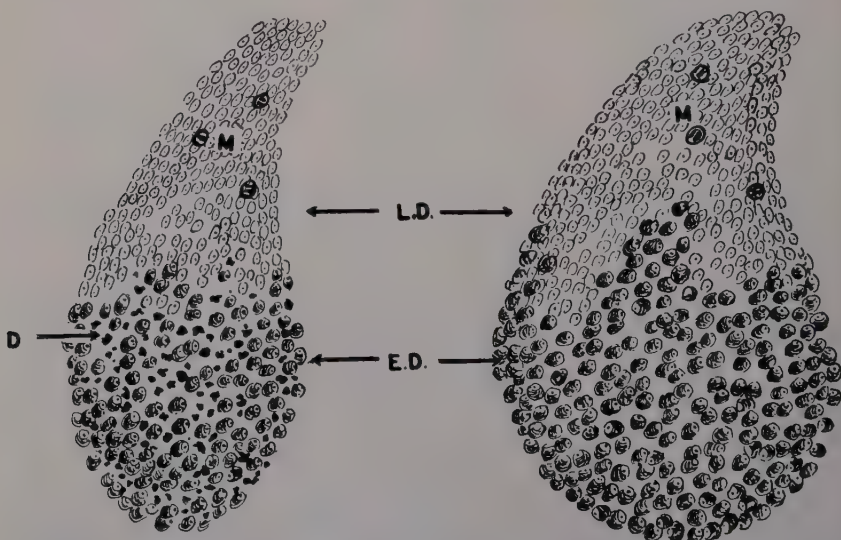


FIGURE 8. Cervical ganglion (left) and brachial ganglion (right) of six-day embryo. D, degenerating neuroblasts. ED, early differentiating, ventro-lateral neuroblasts; LD, late differentiating, medio-dorsal neuroblasts; M, mitoses.

fifth and sixth days, a large-scale degeneration of early differentiated neurons occurs in the cervical and thoracic ganglia, but none occurs in the limb-innervating ganglia (FIGURE 8). Differences were also found in the mitotic activity, and the final regional differences in the size of spinal ganglia are the result of a combination of these two factors. It is of considerable interest to note that the regressive changes produced in limb ganglia as a reaction to limb extirpation are identical with changes which occur in cervical and thoracic ganglia as part of the normal pattern. The overproduction of neuroblasts, with a subsequent partial breakdown, seems to be a rather wide-spread feature in normal neurogenesis. Cell death is known to be a common phenomenon in embryonic development and an integral part of many morphogenetic and histogenetic processes (see Glücksmann, 1951); but its involvement in the creation of numerical differences between cell groups seems to be rather unusual. In this connection, the point should be stressed that, in all instances of cytolysis observed so far in the nervous system, the degeneration affects neuroblasts which have already sent out their neurites. Therefore, the future analysis of the factors which are responsible for the breakdown of nerve cells will have to take into consideration not only the immediate environment of the doomed cells but also the situation at the axon terminals.

Migration

The significance of migrations of undifferentiated neural epithelial cells and of neuroblasts in neurogenesis has been discussed elsewhere (Hamburger and Levi-Montalcini, 1950). Individual cell migrations and cell group migrations are of greatest importance for the establishment of the topographic patterns of nerve centers, and we cannot hope to come to a deeper understanding of the origin of the stratification of the brain and of the spinal cord until we approach the difficult analysis of the factors which are responsible for the activation and the direction of these migrations.

Trophic Relations

The unique property of the nervous tissue to spin out nerve fibers presents to the embryologist a number of special problems. Foremost among them are those related to the directional outgrowth of nerve fibers and to the establishment of the highly selective synaptic connections. A review of this wide field of investigations is not within the scope of the present discussion (see reviews by Piatt, 1948; Sperry, 1951; Weiss, 1941, 1951). We shall focus our attention to some other embryological aspects of nerve fiber outgrowth and synapse formation which may be referred to, collectively, as "trophic correlations", although they represent physiologically heterogeneous phenomena. We shall discuss first the relations between primary nerve centers and non-nervous end organs and then the relations of nerve centers to each other.

Direct Effects of the "Remote Milieu" on Nerve Centers

As soon as the neurite emerges from the neuroblast, a new chapter is opened in the life history of the nerve cell. It reaches out into a new sphere

of influence and, at the same time, it comes under the control of the conditions to which the growing tip of the neurite is exposed. It acquires, so to speak, a second milieu, and some nerve centers become as sensitive to changes in their "remote milieu" as they are to changes in the immediate milieu which surrounds the pericaryon. In many instances, the successful completion of cell differentiation depends on the maintenance of normal trophic relations with the periphery, and the structural integrity of the neuron is threatened if this connection is interrupted.

This trophic dependence on the periphery is not a specifically embryonic property of the primary nerve centers. It persists throughout the entire life span of the neuron. However, it has very significant repercussions on developmental processes in the nervous system, whereas its role in the adult system is overshadowed by the functional aspects of impulse transmission. Furthermore, reactions of the embryonic nerve cells to peripheral changes are more rapid and more radical than those of the adult nerve cell and, finally, experimental embryology offers a variety of experimental approaches which are not readily available in the adult. For instance, in the embryo, one can disturb these relations without cutting the nerve, by the simple expediency of changing the periphery before the nerve grows out. In the adult, one deals largely with regressive changes following nerve transection, and with the restoration of the normal condition; whereas, in the embryo, one can enhance the growth processes beyond the normal range by enlarging the peripheral field of innervation. The chick embryo has proved to be an excellent material for such investigations.

If one surveys the effects of the decrease or increase of the peripheral area on the primary nerve centers, it is found that different centers behave differently. Some are much more sensitive than others. Following limb bud extirpation, the size of the somatic motor centers is reduced already in early phases of its differentiation (Hamburger and Keefe, 1944), and a hypoplasia, amounting to 90 per cent, can be obtained within eight days after operation (Bueker, 1943). On the other hand, the sympathetic chain ganglia differentiate normally and remain intact at least up to eight days of incubation following the same operation, and regressive changes do not occur until later (Simmler, 1949). The trochlear nucleus is also formed in the normal fashion and normal size in the absence of the primordium of the superior oblique muscle, and the effects of the operation are limited to the slow disappearance of differentiated cells, at later stages (Dunnebacke, 1952). In the spinal ganglia, cell groups lying side by side respond differently to peripheral changes.

The responses of the spinal ganglia were analyzed in detail, and it was found that the hypo- and hyperplasia, following the decrease or increase, respectively, of the peripheral area are the result of a combination of different factors (Hamburger and Levi-Montalcini, 1949). We have distinguished between the responses of differentiated neurons, which are connected directly with the periphery by their neurites, and the responses of undifferentiated cells, which are affected indirectly. We shall discuss the former reactions first.

In the spinal ganglia, one can distinguish two groups of neuroblasts by their topographic position and the time of their differentiation: a ventro-lateral group which differentiates very early and rapidly, and another group which begins to differentiate later and develops more slowly and which occupies the central and mediodorsal regions of the ganglion (FIGURE 8). During the greater part of the incubation period, the former cells are conspicuously larger than the latter, but this size difference disappears later. In the absence of the limb, a considerable number of early differentiating neuroblasts differentiate and form neurites, but shortly after they have done so, that is at 5 and 6 days of incubation, they undergo a sudden and rapid degeneration, and most of them have disappeared completely by 8 days. The late-differentiating cells also undergo their initial differentiation in a normal fashion. During the later part of incubation, they undergo a slow atrophy but never a complete cytolysis. In the reverse experiment of the enlargement of the periphery by implantation of an additional limb, one might expect a cellular hypertrophy. However, cell size differences were not obvious and no cell measurements were made. Later on, a much more powerful promoter of the development of spinal ganglia was discovered in mouse tumors (Bueker, 1948), and, in this instance, we have observed not only a striking cellular hypertrophy but also an acceleration of the differentiation process (Levi-Montalcini and Hamburger, 1951, see FIGURE 9).

Embryologists resort occasionally to the "time pattern" of developmental processes in order to explain differential responses of primordia. The differences which one finds in the time of development of different nerve centers would make such an interpretation particularly attractive in the present instance. Our experiences with tumors, however, indicate that very subtle biochemical affinities exist between different types of nerve fibers and different peripheral structures (see the contribution of Levi-Montalcini in this monograph), and such differences seem to be of much greater importance for the interpretation of the different reactions of nerve centers than time patterns.

The trophic relation between the embryonic nerve cell and its "remote milieu" has its counterpart in the adult nerve cell. We recall the regressive changes in neurons following nerve transection and the atrophic and degenerative changes which nerve centers undergo if they are permanently separated from their end organs. The parallelism is even more striking if the regenerative process is compared with the process of embryonic differentiation. Recent studies of P. Weiss and his collaborators and of J. Z. Young and his collaborators have shown that the end result of nerve regeneration is very different, depending on whether or not the regenerating nerve is allowed to re-establish normal terminations in the end organ (P. Weiss, 1951; J. Z. Young, 1948, 1951). One finds, for instance, that in a regenerating motor nerve which is prevented from entering the muscle, the caliber size of fibers is smaller than in a nerve which has re-entered the muscle. "Maturation by increase in diameter and reduction in number of fibers only proceeds when contact with terminal sense organs and muscles is allowed. . . . Although new material is presumably produced by the cell

body, the amount produced is profoundly affected by the periphery" (J. Z. Young, 1948, pp. 68, 69). Young then continues: "Some message or influence must be transmitted up the nerve fibers from muscle or sense organ to produce this change." We think that it is not necessary to assume the transport of some agent from the end organ to the pericaryon. Since the neurite is part of the nerve cell, a direct metabolic exchange between its terminal part and its surroundings would certainly change the physiological condition of the entire cell. Another alternative would be based on the

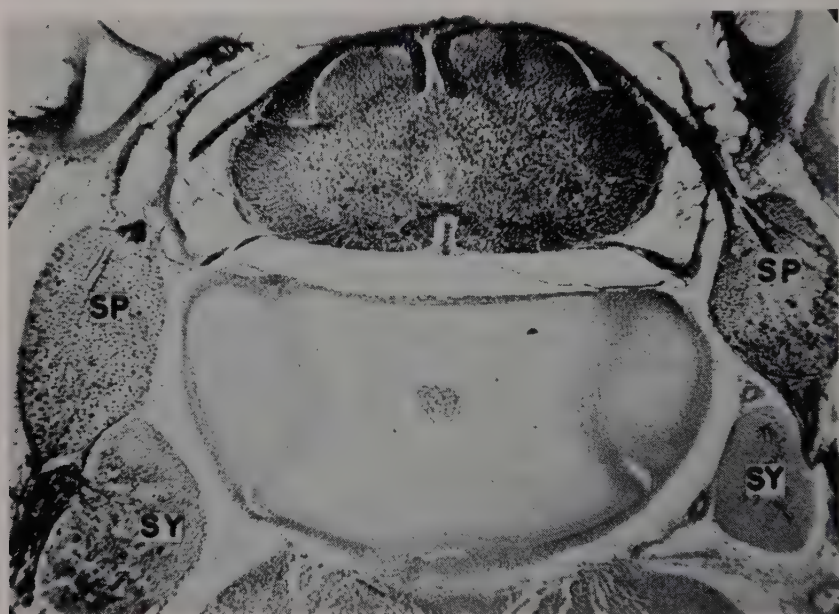


FIGURE 9. Embryo of 11½ days, with intra-embryonic tumor which is invaded by nerve fibers from right (apparent left) spinal ganglion (sp) and sympathetic ganglion (sy). Note hyperplasia of ganglia and hypoplasia of lateral motor column. (From Levi-Montalcini and Hamburger, 1951, *J. Exp. Zool.*, Vol. 116, Figure 13.)

findings of Weiss and Hiscoe (1948) that a continuous production of axoplasm by the pericaryon and its continuous flow along the neurites is part of the normal physiological activity of the neuron. An inhibition of the terminal growth process, by mechanical or other means, would then suffice to upset the metabolism of the cell.

The studies of Edds (1950) and Hoffman (1950) have revealed another interesting aspect of trophic nerve-muscle relations. These authors found that if a muscle is partly denervated, the residual motor nerve fibers begin to develop subterminal collateral sprouts which are guided to adjacent denervated motor endplates. In this case, the disturbance of a trophic equilibrium at the periphery reactivates the growth process of a nerve.

To summarize: The nerve cell establishes a subtle metabolic equilibrium with its "remote milieu" through its neurite. The relation is of consider-

able consequence for the physiology of nerve cell growth, differentiation and regeneration, and its maintenance is a major concern of the neuron throughout its life span.

Indirect Effects of the Periphery

In the embryo, the peripheral milieu has additional, indirect effects. It influences, in some instances, the proliferative activity and the early differentiation of undifferentiated cells and controls in this way the quantitative development of entire nerve centers. The effects on mitotic activity were demonstrated directly by mitotic counts for spinal ganglia (limb extirpation, limb and tumor implantation) and inferred from cell counts, in the case of sympathetic ganglia (tumor implantation). On the other hand, no such effect was found in the basal plate of the brachial spinal cord which gives rise to the somatic motor system (Hamburger and Keefe, 1944), nor in the trochlear system (Dunnebacke, 1952). Negative results, however, are not always unequivocal. It is conceivable that one would obtain positive results under other experimental conditions.

The cells which are formed in excess of the normal complement, in cases of peripheral overloading, appear later on as supernumerary differentiated neurons, as was shown by cell counts in spinal and sympathetic ganglia, using silver impregnated material. From this we conclude that the indirect effects of the periphery extend also to the process of initial differentiation of undifferentiated cells into neuroblasts.

We have referred to these effects which are transneuronal, and, in a sense, supercellular, as "indirect" effects, because their targets are cells which have no direct connections of their own with the periphery. There is evidence to show that those neurons which differentiate first and which do establish peripheral connections are the mediators of this "remote control." The latter would be affected first and their cell bodies, in turn, would influence adjacent undifferentiated cells. This effect would spread in the fashion of an "assimilative induction" (Barron, 1943, 1946; Hamburger and Keefe, 1944).

Intracentral Interactions

Finally, we shall discuss the mutual relations between nerve centers during neurogenesis. We have much less information on this matter than on the peripheral relations; and we shall discuss only a few points which seem to be well established.

It has been postulated that outgrowing fiber bundles create an electric field around them, and thus stimulate the differentiation of neurites and dendrites in cell groups which they pass along their route. The direction of outgrowth of these processes would be perpendicular to the fiber bundles ("Stimulogenous fibrillation," Bok, 1915). This contention was ruled out by three different types of experiments, all of which show that long-range longitudinal fiber tracts have no such effects. Bueker (1943) isolated pieces of the neural tube of $2\frac{1}{2}$ -day embryos by transplanting them to the flank. Levi-Montalcini (1945) eliminated descending tracts in the cord by extir-

pation of a number of cervical segments; and Hamburger (1946) blocked the entrance of both ascending and descending fiber tracts to the brachial cord by insertion of pieces of tantalum foil. In no instance were proliferation, initial differentiation or growth of the respective parts of the cord affected. Short-range local fiber tracts are equally ineffective (E. Wenger, 1950).

Do outgrowing intracentral tracts have any control over the development or maintenance of those centers towards which they grow, and in which they establish their terminal synaptic connections? There is no doubt that such effects are wide-spread. For instance, the extirpation of the limb bud affects not only the primary sensory and motor centers. The secondary sensory centers in the dorsal horn and the intermediate gray matter also become hypoplastic (Hamburger, 1934; Bueker, 1947). Similar transneuronal effects occur in the mammalian embryo (Barron, 1944). We refer also to numerous experiments in Amphibia which show the repercussion of the extirpation of sense organs on secondary sensory centers (reviews in Detwiler, 1936; Piatt, 1948). For the chick embryo, we have a detailed analysis of the effects of otocyst extirpation on the cochlear and vestibular centers (Levi-Montalcini, 1949). This operation removes also the eighth ganglion and deprives the medullary centers of their afferent root fibers. The vestibular centers remained unaffected up to the end of incubation, with the exception of the nucleus tangentialis. The cochlear centers underwent a normal initial differentiation up to eleven days, but, in later periods, they showed hypoplastic effects in varying degrees, which resulted, in part, from an arrest of differentiation and, in part, from a regression and breakdown of neurons. An interesting quantitative relation suggested itself. Those centers were most severely affected which receive no other synaptic connections than afferent root fibers, whereas centers which receive synapses from other sources in addition to root fibers are affected less severely, or not at all.

It seems that, at least in this group of secondary sensory centers, the important first phase of development which establishes the centers is not controlled by incoming fiber tracts, while the neurons require the presence of synaptic connections for their maintenance. In one exceptional case, namely, that of the nucleus tangentialis, the initial differentiation and migration of its component cells were blocked by the absence of the afferent root fibers and this center failed to form altogether.

These investigations which require supplementary studies of other systems indicate that trophic relations exist between nerve centers and that they are as varied and as complex as those between nerve centers and non-nervous end organs. Again, we refer to parallel conditions in the adult nervous system.

In conclusion, we wish to emphasize that all correlations which we have discussed reveal properties of the nervous system which are distinct and separable from its functional property of nerve impulse transmission. In fact, most of the early embryonic interactions occur before the involved nerve centers enter their functional phase. Neurogenesis does not make

use of impulse transmission and its electrical and other correlates as an instrument of differentiation.

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FUNCTIONAL PERSISTENCE OF EMBRYONIC DETERMINATIONS IN FEATHERS AND LATE DEVELOPMENTAL STAGES IN SPURS*

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The plumage coat of fowl, as in other birds, is divided into feather tracts that are separated by predominantly bare areas of the skin, the apteria. The various tracts become apparent in the embryo as each one becomes gradually peopled with the embryonic papillae. The spread of the papillae is for the most part from within the tract toward the periphery until the characteristic number is laid down. Separating sections, equivalent to the apteria of later developmental stages, are not invaded during the process, indicating clearly that segregation within the embryonic epidermis to set apart the prospective feather tracts precedes the local determination of the papillae.

The main features of the origin of the papillae and the development of the definitive feather are taken in outline from a critical survey of the experimental analysis of feather development by Lillie.¹ This paper is a beautiful introduction to the field of study and to the fundamental concepts guiding the investigations.

The embryonic papillae arise as superficial mesodermal thickenings having a thin epidermal covering. Thereafter, the papillae gradually come to lie beneath the skin, occupying the bottom of follicles lined with epidermis and open to the surface. The process is completed by the time of hatching. Definitive feathers arise from the papillae, each of which proliferates a cylinder having an ectodermal wall and a mesodermal core which becomes the pulp (FIGURE 1). The ectodermal wall is composed of three layers, the external one forming the sheath, a middle layer from which the feather proper derives and an innermost layer surrounding the pulp. The three ectodermal layers arise from a thick ring of embryonic cells, the collar. The rhachis, or shaft of the feather, develops along the dorsal wall of the cylinder and is parallel to its axis. Growth is from the base, the apical sections being always the older, therefore. The barbs, which form one vane-half each of the feather, originate as right and left limbs of the ventral triangle that occupies a position in the cylinder more or less opposite the rhachis. Each barb grows at its base to its full length while moving dorsally across the collar to its junction with the rhachis, where growth terminates. A cross section immediately above and parallel to the collar therefore cuts through barbs in all serial stages of development from apex to base.

Visible reactions in the feather germ are confined to a narrow transverse zone, just above the collar proper. Any induced changes from the normal pigmentation, for example, will appear there. Such reactions, and the entire topography of growth of the feather germ are readily observed in so-called split preparations (FIGURE 2; FIGURE 3A). If such a stimulus is of

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

sufficiently brief duration, the feather elements will shortly revert to normal and the band of foreign colour will be moved upward and away from the collar with the continued apically directed growth of the cylinder (FIGURE 3B). In the fully formed feather this same band will appear as a bar extending across the feather vane (FIGURE 3C). The procedure may be repeated at intervals throughout the period of regeneration. The feather will then record in alternate modified and normal bars the history of the previous treatment.

Throughout post-embryonic life, each feather is shed and renewed at



FIGURE 1. Diagram of organization and growth of the feather germ. At the left is shown an empty follicle from which a permanent feather has been removed; the persistent papilla occupies the bottom of the follicle. Next shown is an early stage in the regeneration of the feather germ which at first is wholly enclosed within the follicle. At the right the feather germ is drawn emerging from the mouth of the follicle and extending above the surface of the skin. All growth is from the base and directed apically.

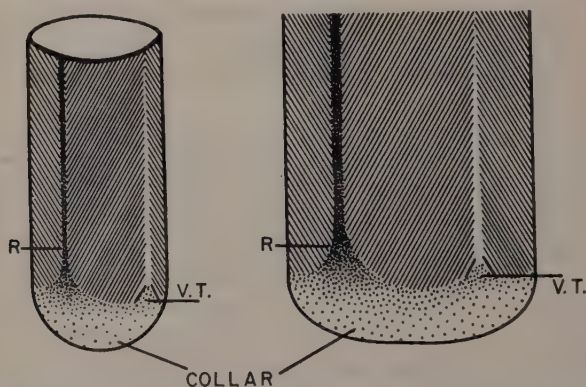


FIGURE 2. The feather germ (apex truncated) is represented diagrammatically at the left. R., rhachis; V.T., ventral triangle. The array of barbs along the collar between one leg of the ventral triangle and the apposed rhachis surface will form a section of the right vane-half of the definitive feather. The barbs extending across the opposed collar limb will form a corresponding section of the left vane-half. The same feather cylinder is slit longitudinally and fixed while flattened under slight pressure to obtain the split preparation. Drawn as a diagram at the right.

intervals by the repeated surges of activity within the follicle. Upon each of these occasions, growth and organization proceed anew from a largely undifferentiated primordium; within, however, the conditions set in embryogeny and the current physiological milieu. These aspects of its development make the regenerate of particular interest in several fields of investigation. It has proved an elegant tool in the hands of the experimental embryologist,^{2, 3} and a most versatile indicator in studies of circulating hormone levels.⁴ Perhaps the most unusual attribute of the feathering, however, is the possibility of recognizing, at each regeneration, the gradients initiated in embryogenesis. These latter properties are of special interest to the present discussion and they will be considered in somewhat greater detail.

The problem, as followed in a series of studies, was to project causally the manifest properties of feathers of a given location back upon their place of origin in the embryonic tracts. The probable relation of embryonic to post-embryonic events was first suggested by the presence of the antero-posterior and transverse axes of the gradients, which were found to distinguish the breast and saddle tracts of the adult plumage. Even more suggestive was the observation that the axes of symmetry were frequently tract-specific, failing to coincide with the major planes of symmetry of the body.

In the following analysis, it was assumed that the axes manifest in the properties of regenerating feathers of the adult plumage tracts reflected the continued action of embryonic gradients. It was further assumed that the embryonic papillae arise as functions of these gradients and the orders of appearance of the papillae accordingly define the embryonic axes. This

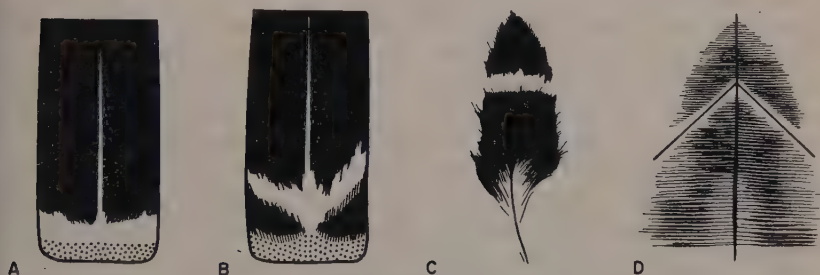


FIGURE 3.

A—Split preparation showing an estrogen-induced band (white) extending across the collar and the barb bases.

B—Split preparation of a similar feather germ, two days later. The induced band had been moved upward with the growth of the feather cylinder; recently formed, basal sections had reverted to normal.

C—The final appearance of the induced collar band as a transverse bar in the fully formed feather.

D—The location of this bar is shown in a mounted feather (barbs fixed in parallel series at right angles with the rachis). The slight apical deviation of the bar from the isochrone (the straight line forming an angle of 45° with the rachis and cutting across each vane-half), indicates that onset of the stimulus was not simultaneous. (A and B, re-drawn from Lillie and Juhn, 1932; C and D, re-drawn from Fraps and Juhn, 1936.)

interpretation called first for a systematic mapping of the sequences in which the papillae are formed in the embryonic tracts, and secondly, for a similar mapping of morphological aspects and physiological properties of feathers in corresponding plumage areas. For the latter purpose, it was obviously important to devise methods whereby feathers could be circumscribed in numerical terms, making it possible to identify any one feather with respect to another of a given location.

The antero-posterior axes were outlined in the growth curves of the feathers lying upon them. Measurements of linear increments showed that feathers along these axes posteriorly exhibited well-defined, gradually increasing rates of regeneration and growth.⁴

The axis of symmetry in a tract is recognized in the symmetry of its feathers and in the adverted asymmetry of its neighbors on the transverse axis. The symmetry of a feather is decided in the similarity of the right and left vane-halves; the degree of its asymmetry in the extent to which the vane-halves vary. The vane-halves, as described earlier, are composed

of barbs which originate from the right and left surfaces, respectively, of the ventral triangle. Barb lengths and the spacing of barbs at the rhachis were adopted as criteria of feather asymmetries and were taken to reflect antecedent differences obtaining during the formation of the opposite sides of the feather.

Certain technical difficulties in this approach became immediately apparent, notably that of obtaining barb lengths without detaching the individual barbs from the rhachis which could have introduced errors. These complications were overcome with the aid of suitably devised techniques. Heavy sheets of cardboard were prepared having a central tapered groove into which the shaft was pressed. The barbs were then brushed out at right angles to the rhachis in thin layers of hot paraffin. The rapidly cooling paraffin affixed the feather to the surface of the board in this position. Such preparations made possible measurements *in situ* of barb lengths and the spacing of barbs with satisfactory accuracy.

Studies of the topography of induced pigment bars in such whole mounts of feathers, when compared to events in the feather germ, lead to observations important in feather development. When a band is simultaneously induced across the entire reactive zone of the collar, it appears in each vane-half of the mounted feather as a straight line forming an angle of 45° with the rhachis at its point of origin and extending across the entire array of barbs (FIGURE 3 D). In its course, the band cuts through all sections of the barbs and the section of the shaft that were present in the reactive zone of the collar at the time of its effect. Each such line is thus a projection of points of simultaneous origin in the collar and has been termed "isochrone" for this reason. Established asymmetries in feather sections bounded by parallel isochrones are therefore true measures of previous, co-existent developmental differences between the vane-halves.

Differences of development and growth between feathers and within feather sections play an important rôle in the expression of any modifications to which the system is susceptible. For example, a certain dose of estrogen adequate to only partial feminization of the most slowly growing feathers will show a progressively diminishing effect along the antero-posterior axis. The same reaction is true of the sub-elements; an induced mark may be the more readily restricted to one vane-half as the asymmetry of the feather is greater. The hormone here serves well as an indicator of developmental properties of the feather, properties which are, as postulated, a continued expression of gradients first recognized in the embryo.

The gradients described by the forming papillae were ascertained for the majority of the embryonic tracts by Holmes.⁵ The first indications of the future breast, thigh, shoulder and ventral tracts appear during the fifth day of incubation. The dorsal tracts commence to appear somewhat later, about the sixth day; the tracts of the future primaries and secondaries between the sixth and seventh day of incubation. A comparison of the findings in the dorsal and the breast tracts will show sufficiently the importance of this study, which should be consulted for more complete details.

The saddle is an example of an unpaired tract (FIGURE 4) where the primary axis coincides with the main antero-posterior axis of the body. The

first series of papillae are formed along this line. Subsequently, papillae are added to the right and left opposite the spaces between the primary papillae. The process is repeated in both distal directions until the full

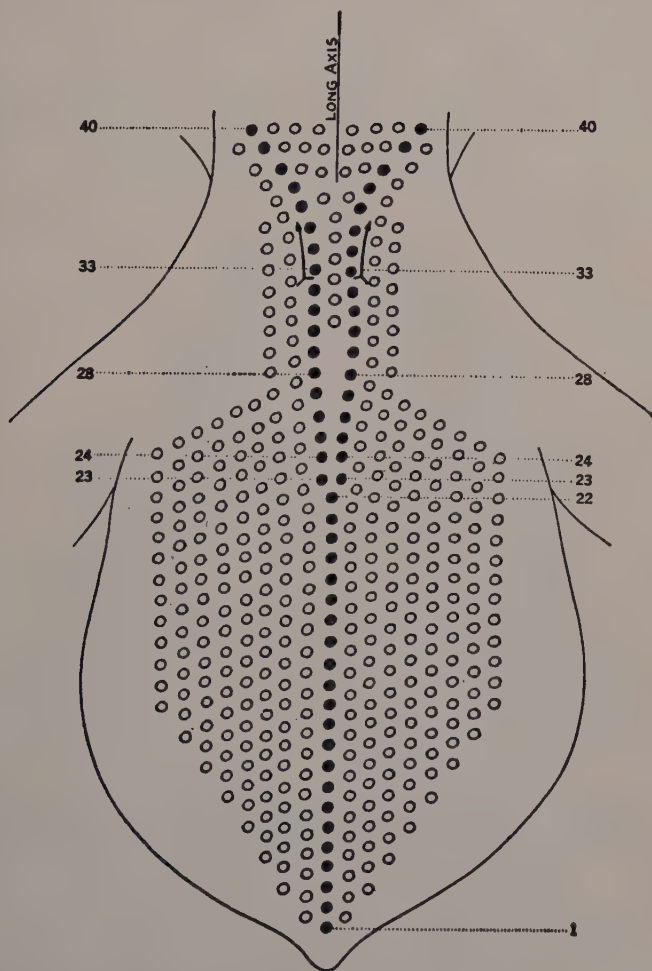


FIGURE 4. Diagram of the dorsal tracts. (Saddle, 1-22; back 23 to ca. 28; Cape, ca. 29 to ca. 32; hackle, ca. 33-40.) The black discs indicate the papillae of the line of origin of the tract. Those extending from the posterior end of the tract to base of arrow are papillae arising simultaneously; the arrows give the direction of the extension of the line. The circles to the right and left of the line of origin are the lateral papillae arising in order. (From the original of Figure 3, by Anne Holmes. *Am. J. Anat.* 56: 522.)

number is laid down. The back is an anterior extension of the saddle, characterized by two adjacent parallel rows of papillae that arise simultaneously. These parallel rows move gradually apart in the cape and papillae are added in the intervening space as well as laterally.

The breast is composed of paired tracts, lying to the right and left of the ventral mid-line (FIGURE 5). The first papillae to arise along the primary tract axes show these to be excentrically located and to parallel, in their

antero-posterior extension, the main long axis of the body. The next papillae to form are laid down to the right and left of these primary tract axes, opposite depressions between the primary papillae. Two more are similarly added in the direction of the distal tract margin and four more toward the opposite side. Counting in transverse rows of each right and left breast tract, the papilla nearest the mid-line as number 1, the distal-most, becomes number 9. The papillae of the primary tract axes always occupy position 6. Where the transverse rows fall short of the more characteristic number of papillae, it is at the expense of lateral papillae, which are not developed.

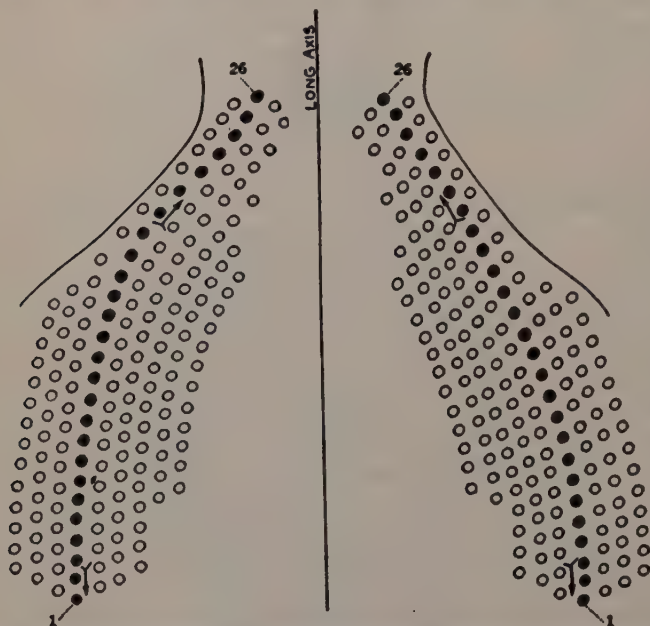


FIGURE 5. Diagram of the breast tract. The black discs indicate the papillae of the line of origin of the tract. Those enclosed by the arrow bases are papillae arising simultaneously; the arrows give the direction of the extension of the line. The circles to the right and left of the line of origin are the lateral papillae arising in order. (From the original of Figure 5, by Anne Holmes. *Am. J. Anat.* 56: 525.)

The above excerpts will illustrate the clarity of the embryonic processes. With this foundation, a specific example of the rôle of these embryonic events in the development of adult feather patterns will be discussed in the case of the breast tracts. Individual feathers of position 1-9, in transverse rows of breast tract, were characterized in the attributes selected as criteria. Barb lengths measured at 0.5 cm. intervals in opposite vane-halves showed the least difference in feathers of position 6, on the original tract axes. Feathers 5 and 7, to either side, proved asymmetrical and mirror images. The same asymmetry continued in increasing degrees in feathers of successively more lateral positions.⁶

A later study,⁷ employing the methods described in preceding pages, deepened these first observations greatly. Barb numbers on successive isochrones were symmetrical in feathers 6, and reversed in comparable

collar limbs of feathers 3 and 9. Linear growth in general was most rapid in feathers 6 and those immediately adjacent, decreasing in feathers of the lateral margin, notably in number 9.

The primary axis of the embryonic feather tract is thus seen to continue effectively as an axis of symmetry. It also continues as a region of higher developmental potency, reflected in the more rapid growth of feathers along its axis. It should be emphasized that the measurements outlined in summary were all obtained in the Brown Leghorn male. The breast feathers here are solid black, becoming a soft grey in the basal fluff.

Differences in pigmentation between apical and basal sections of the main feather vane occur, however, in other fowl, often appearing late, at an age when the full adult plumage is being assumed by the bird. These circumstances made the patterns spontaneously developed at this time an admirable further test of the continued expression of the embryonic gradients. In one example, a hybrid male,⁸ the juvenile breast feathers were irregularly barred black and white with a dusting of red. The adult feathers became a pure white with a black tip. The apical spangle was symmetrical in feathers 6, while lateral feathers of opposite sides showed mirror-imaging asymmetries. Henley,⁹ working in Willier's laboratory, demonstrated the identical relations for a pure-bred fowl, the Silver Spangled Hamburg.

It is clear that all analysis of embryonic determinations in later functions is made possible solely in developing feathers, which furnish such sensitive indicators at each regeneration. The feather reflects in its current pattern the growth properties given by virtue of its position in the tract and in its participation in the general metabolism of the body. An experimental alteration of feather growth should accordingly lead to changes of pattern. The agent used in such studies must be free of all direct action on the feather pigments as any such effect would necessarily obscure reactions due to altered rates of feather growth. The drug thiouracil, familiar for its depression of thyroid activity with the ensuing diminution of metabolic rates, proved suitable when used in fowl of appropriate genetic composition. The selection of the birds to be used was guided by the findings reported in earlier studies where surgical techniques were employed. Thyroidectomy is followed by the disappearance of black in the body feathers of certain fowl,^{10, 11} but the black in the barred plumage of the Plymouth Rock is unaffected.^{12, 13} Red pigments also are retained unmodified by the operation.

Fowl possessing the appropriate combination of pigments are first generation males from Barred Rock male—Brown Leghorn female matings. These birds are barred as is the sire, but show occasional red in characteristically basal sections of dorsal feathers. When fed thiouracil, displacements of the red pigments were seen first in the dorsal surfaces. Later periods of treatment were marked by the appearance of red in feather tracts where it was never seen normally.^{14, 15} These results further show the dependence, for localized expression, of feather pigments upon the growth properties of the feather.

Thiouracil also affects the spur of the adult male. Its action in these structures is upon normal processes that, although occurring late in develop-

ment, may well be considered inductive in nature.¹⁶ The spur, as viewed in X-ray films, becomes calcified only when it is already a well-grown appendage of the shank. The first stages of calcification appear at a distal point within the spur. The next stages show the organization of the central core, which progressively extends proximal-ward. While the core is still at a physical distance (as judged from the X-ray photographs) from the tarso-metatarsus, it initiates the proliferation of the socket from the surface of that bone. Thiouracil, when present from suitable stages onward, blocks this induction of the socket and the gap between the core of the spur, and the shank remains permanent.¹⁷ It is of particular interest that a critical limit in the distance separating the core from the tarso-metatarsus determines the reaction to thiouracil. If the core had extended beyond this line, although not in contact with the shank, prior to first treatment with the drug, the later union was not interrupted.

It would be impossible in this place to discuss properly the approaches to the rôle of the gradients that have been made from theoretical points of view and via direct experimentation. Dynamic, persistent gradients of the entire organism in the sense of Child¹⁹ are demonstrable in lower forms where general dedifferentiations can be imposed and subsequent reorganizations take place. In higher forms, the gradients are functional only at certain embryonic stages, in that polarities, thereafter fixed, are determined. The axes of embryonic feather tracts are instances of such polarizations. Although the separate points along the axes are indeed fixed in the derivative properties of the persistent papillae lying upon these axes, the gradients may nevertheless be considered functional. For it was seen that the gradients, apparent at each renewal of the plumage, find primary expression in the changing dynamics of feather regeneration and growth.

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DEVELOPMENT OF THE EXCRETORY SYSTEM

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Two aspects of the development of the excretory organs are of interest, namely, development of structure and of function. The former has been investigated more thoroughly and successfully than the latter.

The structure of the three generations of excretory organs in the chick embryo is too well known to be described here. The mechanism of development has been the subject of much experimental work. The pronephros develops as far as we know by self-differentiation, and never reaches a significant degree of perfection in the chick embryo. There is no indication that it ever has an excretory function. Its duct, however, the wolffian duct, becomes the leading part in the development of the other two generations of excretory organs.

The wolffian duct grows in caudal direction along the blastema of the mesonephros, and opens into the cloaca. Nephrons differentiate in that blastema shortly after the duct has reached it, and establish connections with this duct. Boyden (1927), Gruenwald (1937) and Waddington (1938) have shown independently that by destruction of the growing end of the wolffian duct its further growth can be stopped at any level. No normal nephrons differentiate in the mesonephric blastema wherever the latter is not in contact with the duct as a consequence of this operation; this indicates that the mesonephros does not have the capacity of self-differentiation but needs induction by the wolffian duct. Only a few scattered, vestigial nephrons may form in the absence of the duct, and, in some instances, even this does not happen (Gruenwald, 1942b).

A similar process occurs somewhat later when the permanent kidney or metanephros forms. The inductor in this instance is a branch of the caudal portion of the wolffian duct, the ureteric bud. The metanephric blastema differentiates normally only when the ureteric bud grows into it. When this does not happen, for instance, after the above mentioned experimental destruction of the wolffian duct, not even vestigial nephrons develop (Gruenwald, 1937).

These experimental results show that the nephrogenic tissues depend on induction by the wolffian duct or its branch for their normal differentiation. It is therefore of interest to know whether mesenchyme other than that of these blastemas may also respond by the formation of nephrons when in contact with the same inductors. In another series of experiments (Gruenwald, 1942b), the wolffian duct was not destroyed, but a graft of tissues of another embryo of the same age, containing no nephrogenic tissue, was implanted in the duct's path. If the duct then continued to grow, it could either traverse the graft or deviate from its normal direction and reach tissues of the host with which it is not usually in contact. Examples of either of these events occurred, and in no instance was there an induction of nephrons outside of the normal blastema. That the duct has not lost

its ability to induce was shown by several embryos, in which it returned to the mesonephric blastema after traversing the graft, and again induced normal differentiation.

It should be noted that the mesonephric and metanephric types of nephron formation differ from each other. In the former, few nephrons form rapidly and, at a given level, simultaneously. In the latter, many nephrons develop during a prolonged period of time and the nephrons themselves differ from mesonephric ones in their structure. In a few experiments, it happened that the wolffian duct came into contact with the metanephric blastema, and induced in it the mesonephric type of differentiation.

Some of the above mentioned grafts contained nervous tissue which happened to develop in contact with the host's nephrogenic tissue. Whenever this occurred, mesonephric nephrons developed from either the mesonephric or the metanephric blastema. These nephrons were less regular in their development and arrangement than those induced by the Wolffian duct, but much more numerous and better differentiated than those which occasionally form by self-differentiation in the mesonephric blastema. Some of them had tubules which attached themselves to the nervous tissue. This was confirmed in subsequent experiments in which nervous tissue was intentionally placed in contact with the nephrogenic tissue (Gruenwald, 1943). Nervous tissue apparently is very similar to the wolffian duct in its action upon nephrogenic blastema. This has also been found in an amphibian, *Rana fusca* (Van Geertruyden, 1946).

The action of the Wolffian duct upon the mesonephric blastema, according to the foregoing, is to some extent that of an evocator, not strictly specific, since nervous tissue has a similar effect. An entirely normal mesonephros, however, has never been seen in the absence of the wolffian duct, so that some determination of a pattern must be credited to the duct, beyond the action as an evocator. This latter action is even more obvious in the case of the ureteric bud, which alone is capable of inducing normal development of the complex metanephros, whereas the wolffian duct or nervous tissue only introduce irregularly arranged mesonephric nephrons in the same tissue (FIGURE 2).

The Wolffian duct has a profound influence on the development of another part of the urogenital tract, namely the müllerian duct. In all experiments in which the wolffian duct was interrupted, it turned out that the müllerian duct stopped growing in caudal direction at the same level at which the wolffian duct had stopped. Even in those instances in which experimental destruction of the wolffian duct had not reached the growing end and had only interrupted the duct without preventing its further caudal growth, the müllerian duct ended at the point of interruption and was never found to cross the gap in the wolffian duct. Since the müllerian duct grows by multiplication of its own cells rather than differentiation of cells *in situ*, it was improbable that a process of induction was involved. Careful examination of serial sections, particularly with the use of silver impregnation of lattice fibers, revealed that the müllerian duct grows in such a manner that its growing end is contained in a common basal membrane with the wolffian

duct. The progress of this growth in caudal direction is followed by a process of separation of the two ducts, in which part of the cells of the müllerian duct are transformed into mesenchyme and only the remainder forms the definitive duct (Gruenwald, 1942a). It is thus obvious why the müllerian duct cannot grow beyond the wolffian duct. It is caught within

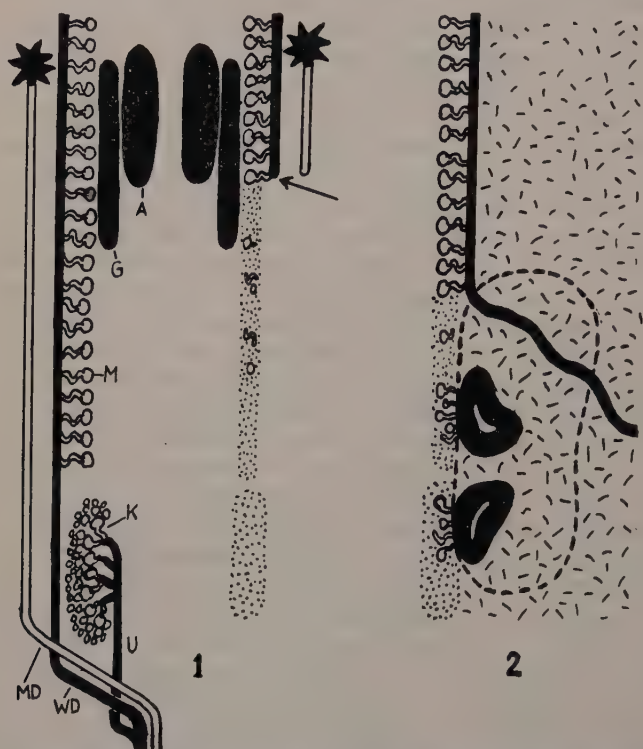


FIGURE 1. Diagram of the developmental relations of the urogenital organs. Those organs which, as far as we know, develop independently of others are shown in solid black, and those which depend on others in thin outline. The left side illustrates normal development, and the right side shows the effects of cessation of growth of the Wolffian duct at the point indicated by an arrow. The undifferentiated nephrogenic tissues are stippled. A, adrenal cortex; G, gonad; K, permanent kidney; M, mesonephros, MD, Müllerian duct; U, ureteric bud; WD, Wolffian duct.

FIGURE 2. Diagram of abnormal relations following the introduction of a graft (outlined by a broken heavy line) containing no nephrogenic tissue, but some nervous tissue (solid black). The Wolffian duct has no inducing effect on mesenchyme of the graft, or the host's body wall (short thin lines). Nervous tissue of the graft induces mesonephric differentiation in the mesonephrogenic and metanephrogenic tissues.

the latter's basal membrane, and is apparently unable to grow alone beyond the point at which the wolffian duct ends.

The gonads and adrenals are not influenced in their development by any of the organs mentioned in the preceding discussion. Only their topographic relations may be influenced by defects of the other organs.

A summary of the developmental relations discussed in these pages is presented in FIGURE 1. Those organs which develop independently of others, as far as we know, are shown in the diagram in solid black. Those which depend on any other structures for their normal differentiation or

growth are shown with thin outlines. Normal development is indicated on one side of the figure and on the other side is shown the course of events which follows experimental or spontaneous stoppage in the growth of the wolffian duct at the point indicated by an arrow. It is apparent that this single event produces a series of changes which, in their final aspect, will be different according to sex. It is of interest to note that the same combination of defects which has been produced experimentally in chick embryos by interrupting the growth of the wolffian duct, occurs in a significant number of human embryos as a spontaneous malformation (Gruenwald, 1938). The fact that this particular combination is prevalent suggests that the same developmental relations between the parts of the urogenital tract that have just been described for the chick, also exist in the human embryo.

The details of renal function in the chick embryo are not well known. There are several lines of evidence which suggest the time at which the function of the metanephros and mesonephros begins. Evidence along several lines agrees that function of the mesonephros begins on the fifth day. After experimental interruption, hydronephrosis of the wolffian duct begins to appear at approximately five days of incubation, indicating that secretion has produced sufficient pressure by that time to accomplish this dilatation. Trypan blue, injected into the air chamber of developing eggs has been traced through the excretory organs, and Atwell and Hanan (1926) found it in the mesonephric tubules from the fifth day of incubation until near hatching time. Hurd (1928) found by this same method that the beginning of function progresses in the mesonephros in caudal direction, just as morphological differentiation proceeds. Termination of function of the mesonephros also occurs in the same direction shortly before hatching time. It was mentioned earlier in this conference (Moog) that the distribution of alkaline phosphatase changes from a diffuse one to one which indicated specific function, also on the fifth day of incubation. Chemical examination of the contents of the allantois (Fiske and Boyden, 1926) has not given any information on the inception or development of renal function because elimination of nitrogenous waste products was complete throughout the period studied, again from the fifth day of incubation. Collection of embryonic urine prior to this period has not been practical.

The only evidence which might indicate the beginning of function of the permanent kidney comes from the above-mentioned experiments with trypan blue. This dye was first found in the permanent kidney on the 11th day of incubation. Both mesonephros and metanephros are apparently ready for function without delay as soon as their nephrons are formed. Half a day before the beginning of function the glomeruli are hardly discernible and parts of the tubules are incompletely demarcated from the mesenchyme and have no lumen.

We have no knowledge of abnormalities of renal function, or of morphologic renal damage produced by any of the experimental procedures, radiation, treatment with chemicals, or inoculation of living agents, to which innumerable chick embryos are subjected in our laboratories. It would be

a laborious undertaking, often unrelated to the original purpose of the experiment, to examine in detail the embryos which served as hosts in these experiments. Examination, however, may turn out to be highly rewarding and reveal facts of much interest and significance, not only in relation to the excretory organs.

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ADRENAL CORTICAL-ANTERIOR PITUITARY RELATIONSHIPS DURING EMBRYONIC LIFE*

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Introduction

For the purposes of this report, pituitary-adrenal relationships during embryonic and early post-natal life have been considered from three points of view. These are: adrenal-cortical dependence upon the anterior pituitary; pituitary response to adrenal-cortical hormone in circulation; and the behavior of the adrenal-pituitary system under conditions which would lead to increased cortical hormone production in the adult.

Dependence of the Cortical Adrenal upon the Anterior Pituitary during Embryonic Life

The earliest successful analysis of the significance of the anterior pituitary to the development of the embryo was accomplished by Philip E. Smith (1916 and 1920). After ablation of the buccal element of the pituitary of the tail bud stage anuran, he subsequently observed profound alterations in rate of growth and degree of pigmentation and found the differentiation of gonad, thyroid, and adrenal cortex to be arrested. When hypophysectomized animals were sacrificed at 187 days of age, adrenal cortical elements were found to be reduced both in volume and in the amount of osmophilic material which they contained. Subsequently, Smith and Smith (1923) were successful in preventing these changes by injecting hypophysectomized larvae with fresh beef anterior pituitary extracts. Atwell (1934) achieved similar results with 525-day-old hypophysectomized *Rana sylvatica* and *R. pipiens* by the administration of a purified adrenocorticotrophin. While clearly demonstrating the eventual dependence of the cortical adrenal upon the anterior pituitary in amphibians, the nature of these experiments does not permit determination of the time of onset of the dependence, either within the period of embryonic life proper, *i.e.*, prior to exhaustion of yolk supply, or during the prolonged larval phase.

Studies involving decapitation of the mammalian fetus provide unequivocal evidence that the anterior pituitary is essential to normal adrenal differentiation during embryonic life of these forms. Jost (1948) reports decapitation of the 18- or 19-day-old fetal rabbit causes adrenal cortical atrophy by the time of parturition. Employing similar techniques, Wells (1948 and 1949) has successfully prevented this atrophy in the decapitated fetal rat by repeated administration of adrenotrophin to the fetus.

On the contrary, the evidence relating to embryonic adrenal-pituitary dependence among birds has been entirely negative until recently. Two methods of eliminating the pituitary have been used: localized application

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of X-rays (Wolff and Stoll, 1937), and surgical decapitation (Fugo, 1940). While the studies based on these varied procedures have agreed insofar as adrenal independence of the pituitary is concerned, they differ in other respects. Wolff and Stoll observed that their X-rayed material was entirely normal, aside from the primary X-ray lesion. Assuming the radiation had eliminated the pituitary, they were led to conclude that this organ was functionless during the embryonic life of the fowl. On the other hand, Fugo noted retarded somatic growth and defective differentiation of testis, ovary, and thyroid after decapitation of the early chick embryo. In the light of current knowledge of adrenal-pituitary dependence in the course of mammalian development, and our knowledge of the rapid adrenal atrophy which follows hypophysectomy of adult pigeons (Miller and Riddle, 1942) and domestic fowl (Nalbandov and Card, 1943), these results, as far as the adrenal is concerned, seem unexpected.

A recent re-investigation of the adrenal-pituitary relationship in the chick embryo has provided considerable evidence that the anterior pituitary is indeed essential to normal cortical adrenal development (Case, 1951). A modification of Fugo's decapitation technique was employed, coupled with studies of adrenal weight, ascorbic acid content, and lipid distribution. White Leghorn embryos, stages 11 to 13, according to the normal table of Hamburger and Hamilton (1951), were decapitated at the level of the mesencephalon. Embryos with an eye cup severed in lieu of the mesencephalic cut served as operated controls. Operated embryos were sacrificed at ages from 14 to 19 days. Some adrenals were preserved in ten per cent neutral formalin for histochemical studies. Others were assayed for total ascorbic acid by the method of Roe and Keuther (1943). Liver tissue from control and experimental groups was similarly treated.

Data relating to body weights were found to agree substantially with those of Fugo (TABLE 1; FIGURE 1). A correction for head weight is made (broken line, FIGURE 1), however, which renders the differences between normal and decapitated embryos considerably smaller than previously reported.

Considering the differences due to removal of large amounts of non-pituitary tissue by decapitation it would appear there is no significant dwarfing effect due to the absence of the pituitary prior to the fourteenth day of incubation. This conclusion is further supported by measurements of tarso-metatarsus and longest toe. On the 14th day, these limb elements are 95 per cent of normal and have been reduced to 77 per cent of normal by the 17th day.

Similar conclusions are to be drawn from the adrenal weight data (TABLE 1; FIGURE 2). Adrenal weights of normal and decapitated embryos are not different during the fourteenth and fifteenth days. Adrenal weights of the

* This correction, which is only of an approximate nature, is made as follows:

$$\left[\begin{array}{c} \text{Corrected body weight} \\ \text{decapitated embryos} \end{array} \right] = \left[\begin{array}{c} \text{Uncorrected body} \\ \text{weight decapitates} \end{array} \right] + \left[\begin{array}{c} \text{Head weight} \\ \text{normals} \end{array} \times \frac{\text{Uncorrected body} \\ \text{weight decapitates}}{\text{Normal body weight}} \right]$$

Normal head weight is taken here as weight of head severed by a cut in the plane of the tomium of the mandible, thus corresponding to the average extent of decapitation.

two groups are increasingly divergent after the sixteenth day. Actually, there is no significant change in adrenal weights of the decapitated embryos after this day.

Total ascorbic acid assays revealed characteristic differences between normal and experimental adrenals while liver ascorbic acid levels appeared to have been unaffected by the operation (TABLE 2; FIGURE 3). From the twelfth to the nineteenth days of incubation, normal adrenal ascorbic acid increased from $0.74 \pm 0.10 \mu\text{g.}$ to 9.35 ± 0.59 , an overall rate of increase of $1.2 \mu\text{g.}$ per day. From the fifteenth to the nineteenth day, adrenal as-

TABLE 1
BODY AND ADRENAL GROWTH OF NORMAL AND DECAPITATED
WHITE LEGHORN EMBRYOS

Age days	Body weight (gm.)		Adrenal pair weight (mg.)	
	normal	decapitated	normal	decapitated
12	4.25 ± 0.65 (15)†	—	0.75 ± 0.17 (25)	—
13	6.46 ± 0.38 (27)	—	1.12 ± 0.30 (27)	—
14	9.74 ± 1.23 (16)	7.40 ± 1.52 (11)	1.55 ± 0.39 (20)	1.61 ± 0.30 (11)
15	12.57 ± 1.74 (32)	8.80 ± 2.11 (26)	2.08 ± 0.50 (31)	2.04 ± 0.29 (24)
16	16.15 ± 1.94 (10)	10.03 ± 1.30 (15)	3.19 ± 0.52 (20)	2.32 ± 0.25 (15)
17	18.39 ± 2.63 (17)	12.40 ± 1.83 (17)	3.30 ± 0.76 (17)	2.49 ± 0.38 (17)
18	22.56 ± 3.20 (11)	15.40 ± 2.15 (14)	4.50 ± 0.83 (19)	2.38 ± 0.42 (12)
19	24.54 ± 3.31 (20)	16.00 ± 2.22 (15)	4.76 ± 1.33 (20)	2.48 ± 0.53 (15)
20	28.07 ± 3.32 (12)	17.11 — (5)	6.00 ± 0.91 (12)	2.52 — (5)
21	32.77 ± 3.55 (18)	—	6.38 ± 0.97 (15)	—

* Standard deviation.

† Number of cases.

corbic acid in decapitated embryos varied around 2.5 to $2.7 \mu\text{g.}$, with no significant change apparent. When these data are related to wet adrenal weights, the fifteenth-day value is similar to normal values occurring on the twelfth and thirteenth days. This leads to the speculation that the ascorbic acid values of 12- and 13-day normal adrenals represent a minimal value attained in the absence of anterior pituitary stimulation. In all likelihood this value may represent medullary ascorbic acid, since histochemical observations have shown the chick adrenal medulla develops characteristically high concentrations of ascorbic acid early in embryonic life (Barnett and Bourne, 1942). This implies that the adrenal ascorbic acid mobilizing effects of the anterior pituitary come into play on approximately the thirteenth day. Thus the ascorbic acid data appear to be in reasonably close agreement with the adrenal weight data, producing together a picture of increasing adrenal-

cortical dependence upon the anterior pituitary during the thirteenth to fifteenth days of incubation.

That these ascorbic acid changes are relatively specific to the adrenals is to be concluded from the similarity of the ascorbic acid levels in liver tissue from the two groups (TABLE 2; FIGURE 3).

Although the influence of decapitation upon adrenal ascorbic acid levels is apparently specific, it is unfortunate that data are not available concerning ascorbic acid levels in the adult hypophysectomized fowl. At least

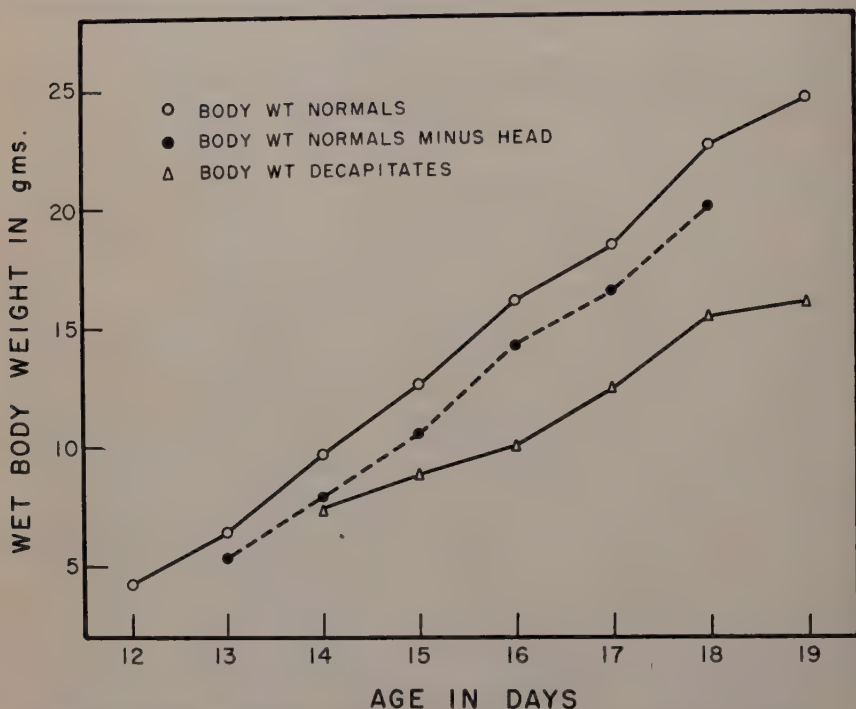


FIGURE 1. Wet body weights in grams, of normal, normal minus head, and decapitated chick embryos.

among adult mammals [except the scorbutic guinea pig (Long, 1947)], adrenal ascorbic acid levels appear to parallel adrenal activity. Birds, however, apparently do not undergo the rapid ascorbic acid fluctuations which characterize acute adrenal activation in the mammals. Young chicks, according to Jailer and Boas (1950), undergo no adrenal ascorbic acid depletion after administration of epinephrine or adrenocorticotrophic hormone (ACTH). These observations have been confirmed on the duck by Zarrow and Zarrow (1950) and on the bob-white quail by Baldini and Zarrow (1951).

The significance of this variation in avian adrenal function is by no means clear. While it may be considered further evidence that perceptible fluctuations in adrenal ascorbic acid levels are not essential adjuncts of adrenal cortical function, any such interpretation must be tempered by the fact that the fowl is probably capable of existing without an outside source

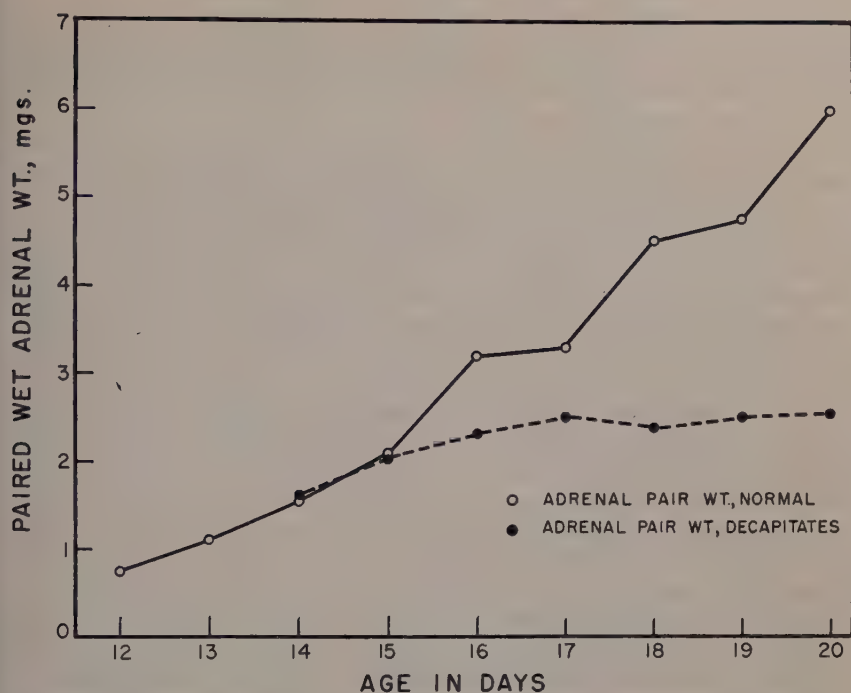


FIGURE 2. Wet adrenal pair weights, in milligrams, from normal and decapitated chick embryos.

TABLE 2
ASCORBIC ACID CONTENT OF ADRENALS AND LIVER OF NORMAL AND
DECAPITATED WHITE LEGHORN EMBRYOS

Age days	Adrenals				Liver	
	Normal		Decapitated		Normal	Decapitated
	Total μ g.	μ g./ mg.	Total μ g.	μ g./ mg.	μ g./mg.	μ g./mg.
12	0.74 ± 0.10 (10)†	0.95	—	—	—	—
13	1.20 ± 0.08 (10)	0.99	—	—	0.20 ± 0.07 (5)	—
14	2.75 ± 0.12 (10)	1.39	—	—	—	—
15	3.98 ± 0.26 (12)	1.30	2.51 ± 0.27 (9)	0.93	0.20 ± 0.04 (5)	—
16	6.08 ± 0.33 (10)	1.64	—	—	—	—
17	6.51 ± 0.24 (10)	1.51	2.48 ± 0.32 (8)	0.99	0.18 ± 0.06 (5)	0.18 ± 0.08 (7)
18	8.50 ± 0.50 (10)	1.65	—	—	—	—
19	9.35 ± 0.59 (10)	1.67	2.72 ± 0.29 (8)	1.10	0.22 ± 0.05 (5)	0.25 ± 0.07 (5)

* Number of assays.

of ascorbic acid (Hauge and Carrick, 1926). Perhaps this apparent biosynthetic capacity of the fowl enables replenishment of adrenal ascorbic acid at rates high enough to preclude detection of ascorbic acid utilization by the techniques currently employed.

The histochemical examination of formalin-fixed adrenals from normal and decapitated embryos,* stained with Sudan Black B,† revealed profound changes in the cortical cords, the amount of lipid-containing cortical material being greatly diminished in decapitated embryos (FIGURES 4 and 5). The volume of lipid-containing cortical tissue in one adrenal of each of two normal male and three decapitated 18-day male embryos was estimated by the paper weight method. In the normal, the lipid-containing cortex comprised 63 per cent of the gland, compared with 32 per cent for decapitated embryos.

It was demonstrated subsequently that the histological aspects of this atrophic condition can be at least partially repaired by injections of ACTH.‡ Three decapitated embryos survived to the eighteenth day after receiving 0.5 mg. ACTH subcutaneously on the twelfth, fourteenth and sixteenth days of incubation. The ACTH, in propylene glycol, was injected subcutaneously in the neck after the embryo had been visualized through an opening in the air space. More frequent injections would have been desirable but the trauma associated with them was invariably found to be lethal by the eighteenth day. On the eighteenth day, the three treated embryos were found to possess larger amounts of lipid-containing cortical material than decapitated controls (FIGURE 6). By the paper weight method, this increase amounted to 45, 47 and 47 per cent respectively (as compared with 32 per cent for untreated decapitated embryos and 63 per cent for 18-day controls). Despite the apparent increase in cortical elements, there did not appear to be any increase in total size of the gland.

It may well be asked why the adrenal differentiates to such an approximately normal extent in the complete absence of the pituitary. While this may represent the initial self-differentiative capacity of the organ, one cannot eliminate the possibilities that the degree of differentiation attained may represent either a permanently pituitary independent component of the adrenal (similar to the glomerulosa of the rat) or the effects of an extra-pituitary supply of adrenocorticotrophin.

Pituitary Response to Circulating Adrenal Hormone

From the foregoing discussion, it would appear there are sufficient grounds to consider the adrenal cortex dependent upon the anterior pituitary, at least during the last half of embryonic life. Next, it is appropriate to inquire whether or not the adrenal itself has an influence upon the anterior pituitary during embryonic life. The direct experimental approach to this

* Crossbred (White Leghorn ♂♂ × Rhode Island Red ♀♀) in addition to White Leghorns.

† Dempsey (1948) has pointed out that the sudanophilic areas of the adrenal are often more extensive than the regions containing ketosteroids. Therefore, in order to characterize more precisely the nature of the deposits in the cortical cords of normal and decapitated embryos, the histochemical procedure of Ashbel and Seigleman (1949) was employed. This method, considered relatively specific for ketosteroids, gave essentially the same picture as was obtained with the less specific Sudan dyes.

‡ Porcine preparation, Armour Laboratories, Chicago, Illinois.

question, adrenalectomy, is considerably more difficult to accomplish in the embryo than is decapitation. It has, nonetheless, been performed on

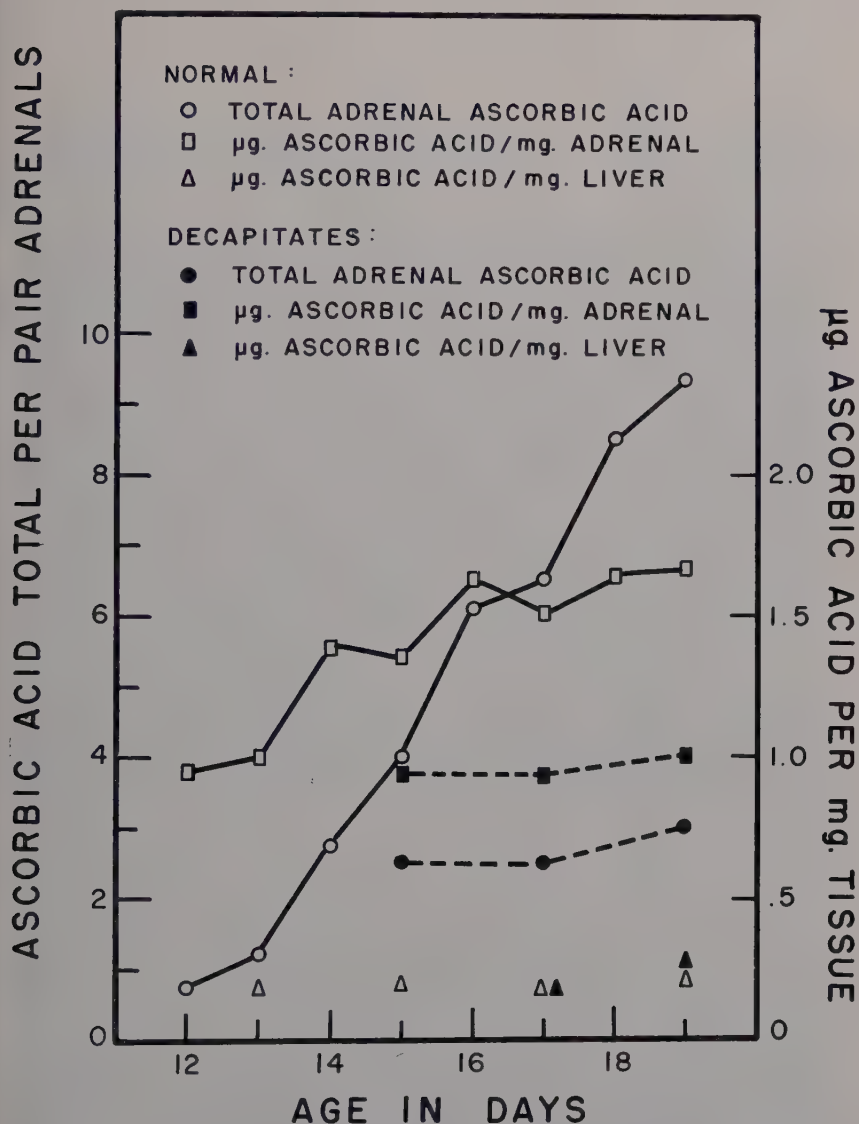


FIGURE 3. Total adrenal ascorbic acid, in $\mu\text{g.}$, and ascorbic acid, $\mu\text{g./mg.}$ adrenal and liver tissues, from normal and decapitated chick embryos.

the fetal rat by two investigators, Tobin and Kitchell. Tobin (1939) destroyed the adrenals of 17-day fetal rats by cautery and noted uneventful development until sacrifice near the time of birth. An advanced state of thymic differentiation and cytological changes in the anterior pituitary were

reported. These involved basophilic degranulation and a decrease in the number of acidophils. In 1950, Kitchell provided stronger evidence of adrenal influence on the pituitary in a study of the effect of unilateral adrenalectomy on the intact adrenal of 19-day fetal rats. Approximately two days after the operation, the intact adrenal was found to have increased 43 per cent in volume.

Since the compensatory hypertrophy of the adrenal remaining after unilateral adrenalectomy of adult rats may be prevented by administration of cortical hormones, it has been generally conceded that the hypertrophy is the result of increased pituitary adrenotrophin secretion in response to

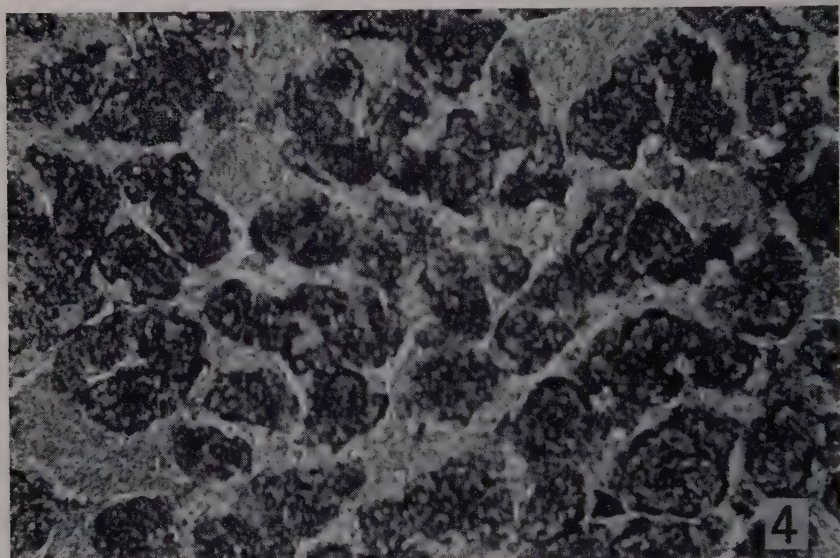


FIGURE 4. Normal adrenal showing intense sudanophilia in numerous large cortical cords. (FIGURES 4-6). Frozen sections of 18-day chick adrenals fixed in ten per cent neutral formalin, sectioned at 20 μ , and stained with Sudan Black B. No counter stain. Initial magnification: 430 \times .)

lowered blood corticoid levels (Sayers and Sayers, 1948). The similarity of these responses in the adult and fetal rat strongly suggests that the fetal anterior pituitary can be influenced by the secretory activity of the fetal adrenal.

Another component, the maternal adrenal, appears to have been added to this system by Ingle and Fisher (1938). They report an increase of 30 to 40 per cent in adrenal weights of term rat fetuses after maternal adrenalectomy on either the seventh or fourteenth day of pregnancy. Since the transfer of maternal ACTH, across the placenta to the fetus, was rendered improbable by the results of fetal decapitation, it would appear that, as Ingle and Fisher (1938) postulated, the stimulus to hypertrophy may arise in the fetus itself. Since there is no reason to presume that adrenal steroids of maternal origin cannot reach the embryo, it would seem that the ACTH release mechanism of the fetal rat pituitary must be adapted

to relatively high cortical hormone levels representing the contribution of both fetal and maternal adrenals. Viewed in this light, the experiment of Ingle and Fisher confirms the essential role of the fetal pituitary in compensatory adrenal hypertrophy in the mammalian fetus.

The author has recently attempted to produce adrenal-pituitary compen-

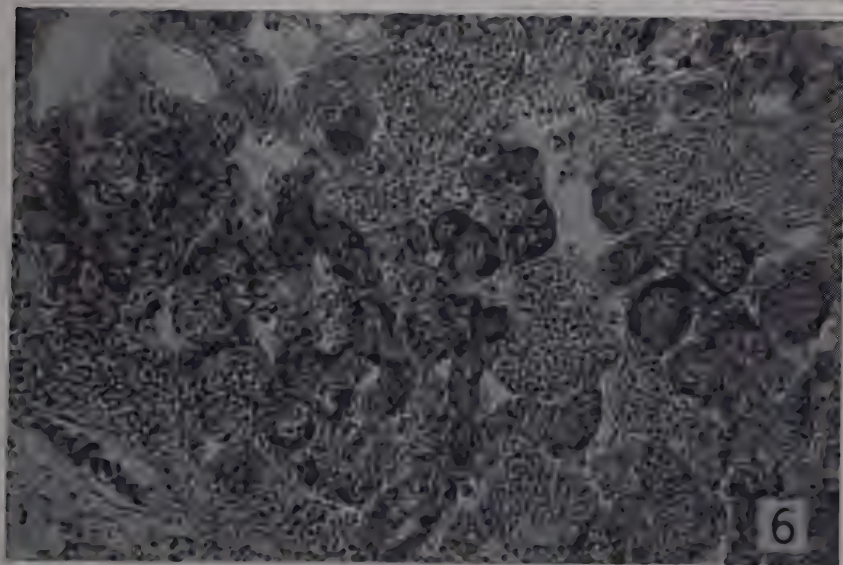
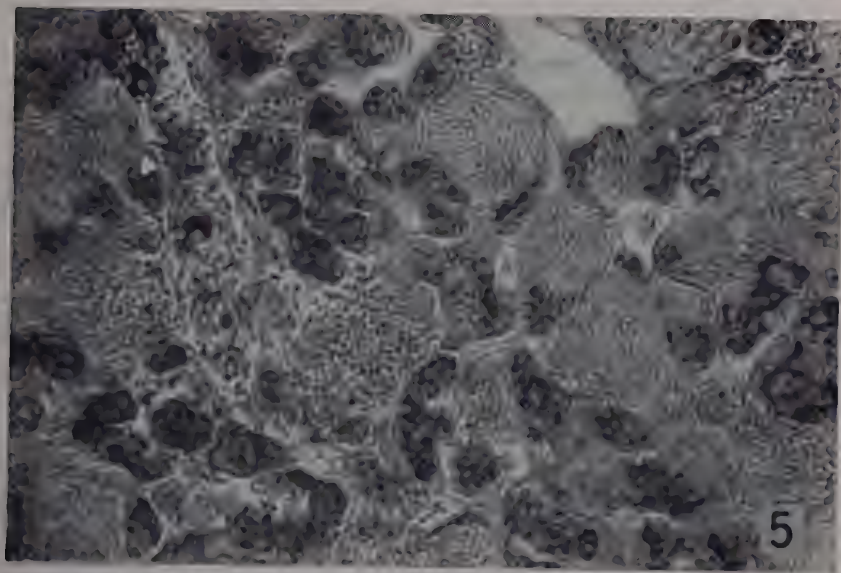


FIGURE 5. Adrenal of decapitated embryo. Note diminished number and volume of sudanophilic cortical cords.

FIGURE 6. Adrenal from ACTH treated decapitated embryo. Intermediate nature of cortical cords is apparent.

satory effects in the crossbred chick embryo by supplying an excess of cortisone acetate from the eighth through the eighteenth day of incubation. An attempt was made to arrive at a dose level which would preserve some semblance of normalcy in the embryo and at the same time be within the range known to be effective in producing compensatory hypertrophy in the adult rat. It appeared that 0.75 mg. per kg. per day was the closest possible approximation of this desired dose. The total dose, 1.25 mg./egg, was injected into the albumen of eight-day embryos. A single dose was employed since Karnofsky, Ridgeway and Patterson (1951) have shown little difference between the effects of divided and single doses of cortisone in the chick embryo. At this dose level, the 18-day mortality was 61 per cent of 100 treated embryos. Body weights of survivors were 60 per cent of normal. In 12 treated embryos, adrenal weights adjusted to body weights were not different from those of water-injected controls. Sudan-Black-B-stained frozen sections presented no sign of abnormal cortical cords. On the basis of the small amount of evidence accumulated so far, it would appear that the chick has not established a reciprocal relationship between the anterior pituitary and adrenal cortex by the eighteenth day. The chance of establishment of this relationship subsequent to the eighteenth day remains to be investigated.

Negative experiments of this type must be interpreted with some caution since there is no assurance that the compound administered is closely enough related to the presumed secretions of the embryonic adrenal to inhibit a pituitary adapted to them. Perhaps a more acceptable method of getting at the problem of the interrelationships of embryonic endocrine glands is to be found in experiments patterned after Etkin's (1936) investigations of the thyrotrophic field of the amphibian anterior pituitary. If a reciprocal relationship exists between the secretory activities of adrenal and anterior pituitary, integrated by secretions not occurring in the adult, one would expect it to be revealed by host adrenal atrophy subsequent to grafting embryonic adrenal to sites closely situated to the host pituitary.

Stress Responses of Embryonic and New-born Animals

Finally, we must consider the ability of the adrenal-pituitary systems of the embryonic and the newly-born animal to respond to conditions necessitating greatly increased output of adrenocortical hormones. The most comprehensive experiments designed to test stress response in the new-born have been performed on mammals. Jailer (1950) estimated the adrenal responses of new-born rats to cold, epinephrine and ACTH by means of adrenal ascorbic acid changes. While the adrenals of the new-born were incapable of responding to epinephrine and cold stress, administration of relatively large doses of ACTH caused adrenal ascorbic acid depletion. Eight days after birth there was a response to epinephrine, and not until 16 days was there a positive response to cold.

Employing different criteria of adrenal activation, Reiss and Halkerston (1950) arrived at a somewhat different conclusion. They have shown that adrenal phosphorus uptake in the adult rat is decreased by hypophysectomy

and is increased by ACTH administration. By the third day after birth, infant rats exposed to low temperatures were capable of increased phosphorus uptake by the adrenals. Since these workers find no direct correlation between the adrenal ascorbic acid decreasing powers of various corticotropic preparations and their ability to increase phosphorus uptake in the adult adrenal, it becomes difficult to arrive at a definite conclusion regarding the ability of the new-born rat to respond to short term stress.

While response to short term stress appears to be somewhat in question, there appears to be no doubt that the new-born human is capable of responding to prolonged stress. Venning, Randall and Gyorgy (1949) have reported that two premature infants subjected to the prolonged stress of atelectasis excreted greater than normal amounts of glucocorticoids. As their lungs cleared, the amounts of glucocorticoids excreted returned to more normal levels. Jailer also mentions unpublished results which imply the ability of the newly born rat to respond to prolonged stress. The nature of these divergent responses to chronic and acute types of stress is at present completely unknown. One can only observe that the experiments concerned with acute stress have employed criteria which, at best, only accompany adrenal activation in the adult, while those relating to chronic stress have employed the actual products of activation as their criteria of response.

Resolution of the problem awaits experiments in which (1) the actual response of the new-born rat to stress is tested, perhaps by cold-stress studies on normal and adrenalectomized new-born animals; and (2) the relative responsivity of new-born and adults to ACTH is precisely determined.

Conclusions

Adrenal dependence upon the anterior pituitary is established fairly early in the embryonic life of birds and mammals. The influence of circulating adrenal hormone upon adrenocorticotrophin release seems to be attained by the mammalian embryo. Although the experiments must be accepted with certain reservations, evidence accumulated so far indicates this relationship does not exist before the eighteenth day in the chick. Acute stress responses may not become established until shortly after birth in mammals, while the capacity to respond to chronic stress appears to be realized sometime before the time of normal birth.

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DEVELOPMENT OF SEX-HORMONE ACTIVITY OF THE AVIAN GONAD

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Since the discovery in 1917 by Lillie of the action of sex-hormones in fetal life of cattle, there have been extensive studies by experimental embryologists on the role of sex-hormones in altering the direction of sex-differentiation of the gonad and associated sex structures in almost all classes of vertebrate embryos. All too often the investigator has been content with the morphological changes induced and too little concerned with such challenging problems as the mechanism of action of the sex-hormone and the development of morphological and functional pattern of gonad as physiological processes. The gonad, like all other characters of the organism, comes into being wholly as a result of physiological processes which are ultimately dependent upon specific physiological agents, the genes.

This paper has as its main objective the analysis of the sequence of events in the development of functional activity of the avian gonad. Particular attention will be given to such problems as the origin and function of the sex-specific tissue elements of the gonad primordium, the time of onset of sex-hormone production, as well as the time of release of sex-hormones into the circulation and their role in regulating the expression of sex-hormone sensitive structures (gonaducts, phallus, *etc.*). Consideration will also be given to the problem of the time in ontogeny when the gonad as a receptor endocrine gland becomes functionally interlocked with the anterior pituitary.

Initial Phases in Gonad Origin. In discussing this topic, care must be taken at the outset to recognize that the ultimate factors concerned are to be sought in the hereditary constitution of the living substance of the avian egg. These factors somehow determine the precise and orderly course of physiological events leading to the fully differentiated gonad, whether testis or ovary. These factors may be thought of as acting differentially in the developing embryo at a particular time and locus, establishing therein the primary conditions for gonad origin. Thereupon follows a progressive series of local coordinated developmental processes which lead initially to the formation of the gonad primordium, comprised of male and female tissue components, and later, through the interaction of which, to the realization of the final specific form and function of the gonad.

It is a well-recognized principle that glands, organs, and other parts of the developing embryo usually arise as localized thickenings or outgrowths from generalized morphological units which have been partitioned off from one or more of the three primary germinal layers of the embryo. In the chick, on each side of its body, the gonad primordium is first apparent as a local thickening of the coelomic wall of the urino-genital ridge, a complex structural unit previously formed by a long series of morphogenetic changes from the mesoderm. Thus the gonad primordium is a modified part of a more generalized embryonic structural part which is ultimately traceable

by backward projection to a particular locus in the mesodermal germ layer. The question next arises as to when and how far back in the development of the mesoderm it is possible to detect the localization of gonad-specific areas, *i.e.*, whether there is for each gonad primordium a corresponding area of specific histogenetic potency for gonad. An analysis of the histogenetic potency of isolates from the flat blastoderm of the chick at early stages (definitive primitive streak and head-process), grown separately as chorio-allantoic grafts, shows that the pellucid area is comprised of localized organ-specific areas, such as the heart, thyroid, and urino-genital complex. The latter area is localized in the region of, and just posterior to, the primitive knot (Willier and Rawles, 1935). It is a single large area of mesonephros-forming tissue, near the center of which is a single smaller area of gonad-adrenal cortex tissue. The time that the urino-genital area becomes bilaterally localized has not yet been ascertained. By analogy with the behavior of other single areas such as the eye and thyroid, it would be expected that, as development proceeds, the single area of urino-genital tendency would likewise become bilaterally localized, *i.e.*, restricted to right and left areas.

Thus, in the chick, the formation of the gonad primordia is preceded by the segregation of an area of specific urino-genital tendency. Its spatial location within the anterior-posterior axis roughly corresponds with the expected definitive position of the urino-genital primordia. Furthermore, the area seems to have a center of strong differentiation tendency which gradually diminishes peripherally to zero, thus exhibiting one of the properties of an embryonic field. These observations are in accord with the well-established principle that the primordium of almost every organ or part of the vertebrate body has its origin in areas having field properties. In this respect, then, the primary phases in the origin of the gonad and its closely associated structures are no different than those of other glands and parts of the embryo. There is no evidence that hormones play a role in these initial phases.

Functional Interaction of Gonad Tissue Components in Sex Differentiation. Once the primary conditions for gonad origin have been established in the urino-genital complex on each side of the body, a progressive series of morphogenetic processes lead to the formation of the gonad primordium. Except as noted below, the primordium thus becomes a composite of two topographically distinct tissue components, commonly designated as cortex and medulla. Into them, a third component, the primordial germ cells, become incorporated. The cortex and medulla typically arise as successive proliferations in the form of sex cords from the germinal epithelium (a local specialized thickening of the coelomic epithelium of the urino-genital ridge). Thus a definite time sequence is seen in their origin from the same primary tissue source. Asymmetrical difference in structural organization of the gonad primordium on the two sides of the body is a characteristic feature in the development of the chick, duck, and many other birds. The left primordium during early stages possesses two tissue components, an outer incipient ovarian cortex and an inner portion, the medulla. The right

primordium, on the other hand, consists typically of a medullary component, only occasionally showing scattered patches of incipient ovarian cortex. These asymmetrical differences in tissue organization are basic to an understanding of the course of events in sex-differentiation.

By tracing the normal course of histological differentiation of the components, it was early surmised that the gonad primordium has a bisexual organization (σ^7 and ♀ tissue elements) on the left side and a unisexual organization (predominantly σ^7 tissue element) on the right side. With the onset of histological sex-differentiation in the female, the incipient ovarian epithelium on the left gonad differentiates into a thickened layer, the ovarian cortex, while the sex cords of the medulla persist but assume the form of epithelial sacs and/or cords. The right gonad of the female becomes histologically similar to the left ovarian medulla, only very occasionally showing small amounts of ovarian cortex. In the male, the incipient ovarian epithelium of the left gonad gradually retrogresses and disappears altogether about the tenth day, whereas the medullary component differentiates into solid testicular cords; the right gonad develops testicular cords identical with those of the left.

Proof that the cortex and medulla are sex-specific tissue components has come from studies on sex-reversal effects produced by sex-hormones (either from testicular or ovarian tissue grafts or by administration of purified or synthetic sex-hormones). In general, irrespective of the sex genotype, androgens tend to bring into expression the male tissue component and to suppress female tissue components; conversely, estrogens tend to bring into expression the female tissue component and to suppress the male tissue component (Willier, Gallagher, and Koch, 1937).

What determines which one of the sex-specific components of the composite primordium will either subside or degenerate, whereas the other will persist and differentiate into the characteristic tissue of the sex-differentiated gonad? Is the fate a resultant of a mutual interaction or antagonism between the sex-specific tissue components? In an attempt to answer these questions, it is first of all evident from theoretical considerations that whether sex-differentiation proceeds in the male or female direction must depend primarily upon a quantitative balance between the opposing sets of male and female producing genes. Which one of the structural components of the bisexual primordium predominates and comes into full expression would be dependent, therefore, upon quantitative differences in the sex-producing tendencies of the genes in the two sexes.

Since the direction of differentiation of the components, irrespective of the sex genotype, is usually altered selectively by male and female sex-hormones and the degree of response depends upon dosage, it is inferred that what the genes actually control from a physiological viewpoint is the quantity of hormone produced. The administered hormone has specific additive effects tending to bring about a reversal of the normal course of differentiation of the two components. Consequently, in normal development, the gonad component with the relatively greater production of sex-hormone determines the direction of sex-differentiation. That hormone becomes the

sex differentiator. Although the genes are the effective sex producers, their influence appears to be mediated by hormone secretion, *i.e.*, by specific sex substances produced by the cortex and medulla (see Spratt and Willier, 1939, Plate III and Willier, 1942).

On the assumption that the foregoing interpretation is valid, it follows that the expression of sex is the resultant of an interaction between medullary and cortical tissue components. A significant example supporting the concept of the interaction of gonad components is found in the common fowl after hatching. After surgical removal or destruction of the ovarian cortex, the ovarian medulla left behind hypertrophies and differentiates into typical testicular tissue (Domm, 1927). The ovarian cortex appears, therefore, to exert an inhibitory influence on the medulla maintaining its tissue in a structurally ovarian form. Whether the medulla has a reciprocal influence on the ovarian cortex is not clearly indicated.

In summary, the gonad primordium, at least the left one, is a composite made up of two distinctive and contiguous tissue elements which differ in sex-specific differentiation tendency and in intensity of sex-hormone production. The gonad component with the relatively greater intensity of hormone production determines the direction of sex-differentiation, the resultant of which is the subsidence or degeneration of one component and the full expression of the other one which is capable of synthesizing estrogens or androgens as the case may be.

Production and Release of Sex-hormone Activity. The evidence and interpretation just presented for the existence in the differentiation process of the gonad of a competitive interaction of contiguous sex-specific tissue components which mediate their effects by quantitative differences in production of sex-specific hormones naturally raises a number of questions. Are the sex-specific hormones different from those produced later in ontogeny? Is the production of sex-hormones merely confined to the gonad at first and later released into the blood stream? When are sex-hormones liberated into the circulation in sufficient quantity to act on terminal or receptor organs (gonaducts, phallus, *etc.*), structures which react selectively to sex-hormones in accordance with their latent characteristic properties?

That the sexually differentiated gonad at later stages in chick development produces sex-hormones which act in a manner similar to that of the synthetic sex-hormones in modifying the direction of differentiation of the gonad primordia has been discovered somewhat recently. By implanting small fragments of a sexually differentiated gonad (6-11 day embryos) into the coelom of a host embryo of 50 hours (preceding by about two days the time of origin of the gonad primordia), Wolff (1947) succeeded in bringing about an approximation of grafted tissue and host gonads and gonaducts. If of opposite sex, the graft tissue modifies the direction of sex development of the reproductive organs of the host. Ovarian tissue causes the formation of ovarian cortex on the left testis. Testis tissue has little or no effect on the left ovary but inhibits or completely suppresses the Müllerian ducts. The degree of sex modification appears to be dependent upon the volume of the tissue graft and the distance between graft and host gonads. In

general, the closer the graft, the greater is the degree of effect. Since the effects produced by grafts of testicular or ovarian tissue are identical in general character with the effects produced by the injection of synthetic sex-hormones, it is apparent that the gonads, within a few days after morphological sex-differentiation has set in, produce specific sex-hormones.

The phenomenon of the local action of the implanted ovarian or testicular tissue on the host gonads and gonaducts has an important bearing not only on the production but also on the time and manner of release of sex-hormones. The observation that the effect is most marked near the source of the hormone and wanes as the distance from it increases is clearly suggestive of a diffusion gradient in hormone distribution. In other words, the manner of dispersal of the sex-hormone from the graft is by gradual diffusion through the intervening tissues with a consequent diminution in its concentration until it finally reaches a level too low to have any influence on the host gonads and gonaducts. Such a diffusion gradient seems to agree with the observed effects, whereas action through the blood stream would not. This phenomenon of local hormonal action is not peculiar to the sex-gland, since considerable evidence has accumulated showing that other endocrine glands, when approximated by grafting, exhibit functional activity prior to the time that the same kind of effect can be elicited through the blood circulation.

The interpretation that the differentiated sex-glands at the ages under consideration produce sex-hormones poses the interesting problem of the time of their release into the blood circulation of normal embryos. Ideal conditions for testing hormonal action at a distance seem to be provided by grafting gonad primordia at sexually indifferent stages to the well-vascularized chorio-allantoic membrane of host embryos of eight to nine days of incubation. At the time this combination is made, the gonad primordia, known to be responsive to sex-hormones (as the injection of synthetic sex hormones into the hen's egg clearly demonstrates), might be expected to react to any circulating sex-hormones of the host embryo for a period of at least twelve days (up to about the time of hatching). Irrespective of the sex-constitution of the host embryo, however, the grafted gonad primordium differentiates in a normal fashion into a gonad of specific sex. No signs of sex-reversal effects are seen. It must be inferred, therefore, that although sex-hormones are produced by the testes and ovaries of the host, as judged by their capacity in coelomic grafts to cause locally sex-reversal of gonad primordia, they are ineffective through the vascular circulation. It would appear that the differentiated sex-glands produce hormones which are either not released or else released in quantities insufficient to bring about any sex-reversal of the gonad primordium in chorio-allantoic grafts. In accounting for the local action, the possibility must also be recognized that the isolation and transplantation of testicular and ovarian tissue somehow disturbs the normal mechanisms of liberation of the accumulated sex-hormones.

When in the course of gonad differentiation are sex-hormones liberated into the blood circulation? The effects of castration by localized X-ir-

radiation of a chick or duck embryo during the sexually indifferent stage indicates that hormone secretions are essential in regulating the direction of differentiation of certain sex-hormone sensitive structures. According to the recent investigations of Wolff and Wolff (1951), an asexual or neutral type of sex structure is obtained after such precocious castration. In both ♂♂ and ♀♀, the genital tubercle and syrinx (duck only) assume the male shape and the oviducts persist. From these results, it is inferred that in the normal embryo the female form of the genital tubercle and syrinx develops only in the presence of ovarian hormone. On the other hand, the retrogression of the oviducts takes place only in the presence of testicular hormone. It may be added that these findings are in agreement with the results obtained by the administration of synthetic sex-hormones to normal chick embryos, the male hormones tending to suppress the oviducts of genetic ♀♀ and the female hormones tending to stimulate the oviducts in genetic ♂♂. If the foregoing facts are correctly determined and interpreted they offer a significant contribution to the subject of sex-differentiation, namely that the female sex-hormone is a differentiator or conditioner of female sex characters in the chick embryo. For a discussion of the evidence that the female sex-hormone plays the role of a conditioner of sexual differences in plumage structure and coloration in certain breeds of fowl, see Willier (1950).

Such castration effects support the view that in the normal embryo sex-hormones are not only produced but are liberated by the early differentiated sex-gland into the blood circulation. It is hardly conceivable that the action of the sex-hormones is mediated in any other way than through the circulation, since structures as far away from the source of the hormones as the genital tubercle and syrinx are affected. Although the nature of the experiments cannot reveal the time that hormone release begins, it may be inferred from the fact that the oviduct in the normal genetic male chick begins retrogression prior to the tenth day and is typically completed by the eleventh day that the hormone secretions in the male chick at least are already in functional operation at that time. The quantity of sex-hormone in circulation is apparently sufficient to regulate the course of differentiation of the receptor sex structures, but in concentrations too low to be effective in altering the direction of differentiation of gonad primordia grown in the chorio-allantoic membrane.

Onset of Gonadotrophic Activity of Anterior Pituitary. The evidence just presented for the existence and liberation of sex-hormones early in gonad differentiation naturally poses a two-fold problem of (1) the extent to which their production and release are dependent upon gonadotrophic stimulation and (2) the time that the anterior pituitary begins to produce gonadotrophic substances as well as to release them into the blood circulation of the embryo.

Whether sex-hormone activity of the gonad during embryonic life is dependent upon gonadotrophic hormones is a problem which is as yet only beginning to be resolved. In the chick embryo after hypophysectomy (removal of the forebrain region) at 33-38 hours, primary morphological sex-differentiation apparently proceeds in a normal manner up to about the

TENTATIVE SCHEME OF INTERRELATION BETWEEN GONAD AND ANTERIOR PITUITARY
IN THE PROGRESSIVE DEVELOPMENT OF FUNCTIONAL ACTIVITY

TIME DAYS	PRIOR TO HATCHING				AFTER HATCHING				
	5 - 7	8 - 9	10	13	18	5	10	30	90
ACIDOPHILES			+	++	+++	+++	-----RELATIVELY SLOW INCREASE-----		
BASOPHILES				+(?)	+(?)	+(?)	+	GRADUAL INCREASE IN NUMBER	
WEIGHT							-----WEIGHT INCREASES 9X-----		
POTENCY				+(?)	+(?)		-----GRADUAL QUANTITATIVE INCREASE-----		
TISSUE ELEMENTS	♂ + ♀	♂ ♀ (♂ LATENT)					-----2-----		
HORMONE ACTIVITY	ONSET ♂ + ♀	LIMITED IN TYPE ♂ OR ♀	PRODUCTION LOW AT FIRST			PROBABLY INCREASES RAPIDLY WITH AGE			
			RELEASE			-----			
			GONADUCTS, PHALLUS, AND SYRINX REACT SELECTIVELY			-----			

13th day, when reductions in intertubular tissue of the testis and of ovarian cortex are first noted. The gonaducts in both sexes, however, develop normally both in time and manner (Fugo, 1940). On the other hand, Ancel (1937) and Wolff and Stoll (1937) report that the entire course of sex-differentiation of gonads and gonaducts proceeds in a normal manner up to the time of hatching in the cyclocephalic chick embryo without hypophysis produced by local X-irradiation at the 12-15 somite stage. By treating such pituitaryless chick embryos with estradiol, Wolff (1937) reports that intersexual males are produced as readily as in normal male embryos. It appears, therefore, that the functional processes involved in sex-inversion are quite independent of hypophyseal action.

Further light may be thrown upon the problem of when the gonad, as a receptor gland, is first responsive to secretions of the anterior lobe by the administration of gonadotrophins. Certain suggestive results have been obtained. According to Domm (1937), in chick embryos examined at 18 days after daily injections of sheep pituitary extracts ("Hebin") beginning at a time just prior to and during the period of histological sex-differentiation (from the fifth to the ninth days of incubation), the gonads of both sexes are hypertrophied, owing to an increase in intertubular tissue of the testis and of medullary tissue in right and left ovaries. No significant advance in the differentiation of the seminal cords or ovarian cortex is observed, however. Unfortunately, whether the gonad has the capacity to respond earlier than the 18th day is not brought out in this study. It would be of considerable interest to ascertain at what time in development the gonad first exhibits a response to administered gonadotrophins.

Although structural changes are produced in the embryonic gonad by the pituitary injection, little or no enhancement of sex-hormone activity is indicated, since neither the comb nor gonaducts in either sex show any significant change from the normal. An enhancement of sex-hormone activity of the gonad by pituitary injections into newly hatched chicks is clearly indicated, however. In contrast to the embryo, a precocious spermatogenesis (without sperm at first) is produced in the testis. Moreover, the testis activity releases sex-hormones, as is indicated by a precocious onset of male sex behavior, an accelerated growth of the head furnishings, and a hypertrophy of the ductus deferens. Although in the female there is no evidence of follicle growth in the ovarian cortex (follicle response may be elicited, however, in still older chicks), enhanced production of female hormone is indicated by the hypertrophy of the oviduct. Curiously, in contrast to the effect produced in the embryo, the right ovary is apparently unmodified. The medulla of the left ovary again shows a pronounced hypertrophy accompanied by a pronounced growth of the comb. Furthermore, the daily administration of gonadotrophins to chicks for ten days, beginning on the fifth day after hatching, readily produces an increase in weight of the gonads in both sexes and an increased growth of the seminiferous tubules and interstitial tissue in males (Breneman, 1936).

These results may be interpreted to mean that the capacity of gonads of the chick to respond to gonadotrophic hormones increases with advance in

developmental age. Although the time that the gonad is first reactive is not worked out, it is clear that it is already responsive by the 18th day, still more so in newly hatched chicks, and probably reaching a maximum in still older chicks. It is thus apparent that the gonads of chicks after hatching have acquired a more marked capacity for precocious growth stimulation in contrast to a much more limited yet definite capacity for induced growth during at least the latter third of embryonic life. Apparently, then, the gonads undergo developmental changes (maturation) which enable them to respond gradually more rapidly to hypophyseal stimulation. Such a view is in keeping with the concept of a gradual increase in responsivity of the gonads to gonadotrophic stimulation after birth in mammals (rat, mouse, *etc.*). This concept was based initially on the finding by Smith and Engle (1927) that the number of implants of anterior pituitary required to induce precocious sexual maturity in ♂ and ♀ rats and mice is roughly inversely proportional to the post-natal age of the host. Subsequent investigations have fully established the validity of this concept.

Is the progressive increase in capacity of the gonad to respond dependent upon gonadotrophic hormones during the normal course of development? Thus far no satisfactory answer can be given to this question so far as the chick is concerned. In sexually immature mammals (from a few days after birth to onset of sexual maturity), however, the removal of the anterior lobe, during the period when the gonad is shown to be responsive to administered gonadotrophins, results in arrested development of the gonad or a retrogression to an immature or infantile state. The amount of retrogressive change is, in general, roughly proportional to the degree of reactivity of the gonad, being greater in older than in younger stages. Since the structural and functional changes following hypophysectomy may be prevented or restored to normal by the administration of anterior pituitary substances (by either implants or extracts), it is clear that the processes of maturation of the gonad in immature mammals are dependent upon the presence of specific hypophyseal secretions. Such secretions are clearly essential to the structural maintenance and function of the gonads beginning early in postnatal life of placental mammals (rat and mouse).

From this short digression to mammals, certain clues are at hand for evaluating the possible significance of the progressive increase in gonad reactivity to gonadotrophins administered to the chick. A plausible working hypothesis is that the increasing capacity to respond is a rough indication of the changing state of maturation of the gonad, as conditioned by hypophyseal secretions. In other words, as the gonad develops, it attains a stage when its further development or maturation becomes dependent upon gonadotrophic activity of the anterior lobe. Dependency after hatching (five to 90 days) is suggested by the occurrence of a correlation with some fluctuations between increase in weight of the testes (accompanied by comb growth) and increase in weight as well as in gonadotrophic activity of the anterior pituitary (Breneman, 1941, 1944, and 1945). A crucial test of this hypothesis, however, would be to determine the effects of hypophy-

sectomy on gonad development and the consequent indirect effects on sex-hormone dependent structures (terminal receptors) at various stages in ontogeny. Unfortunately, the removal of the anterior lobe in the chick, either before or after hatching, is technically quite difficult. Nevertheless, as noted above, Fugo (1940) hypophysectomized chick embryos at 33–38 hours and reported that the course of development of the gonad and gonaducts proceeds in a normal manner up to the 13th day, when retrogressive changes in the gonads are first noted. With respect to time, this finding may not be without significance, since Case (1951a), using about the same stages and a similar method of hypophysectomy, obtained results on the chemodifferentiation of the adrenal cortex which clearly indicate that the pituitary exerts its adrenocorticotrophic effect at least by the 13th day. Using quantitative changes in ascorbic acid and lipids as criteria of adrenal functional activity, he found that these substances increase gradually in normal embryos between the 13th and 17th day but fail to increase in decapitated embryos. Moreover, the administration of ACTH to the decapitated embryos restores the adrenal cortex to normal (Case, unpublished results). Case also noted that, at 17 days, the weight of the testis in relation to body weight was less and the ovarian cortex thinner in decapitated than in normal control embryos.

It becomes evident from these investigations that the anterior pituitary has attained a functional state between the 13th and 17th days. Adrenocorticotrophic activity is clearly shown, but the evidence for gonadotrophic activity is too meagre to be convincing. Means, however, are now available for detecting whether gonadotrophic activity is present, since Case (1951b) has worked out a technique for the successful decapitation of the chick embryo at later stages in development (10–14 days).

The correlation of the onset of gonadotrophic activity with the histogenesis of cell types of the anterior pituitary is a difficult problem to resolve. Only a few investigations have been oriented toward its solution. The time course of differentiation of the chromophilic cells has been studied by Rahn (1939) and Payne (1946). The acidophiles, which are first apparent on the tenth day, gradually increase in number with advance in age, becoming the predominating type of cell by the 18th day (Rahn) or by the third day after hatching (Payne). The time of appearance of the basophiles is less readily determined, owing to a lack of adequate cytological criteria for sharply distinguishing them from chromophobes. A relatively few basophiles seem to be present as early as the 12th day of incubation, however, but large fully differentiated basophiles are probably not clearly evident until about ten days after hatching (Payne) or even later (21 days) according to Rahn. Small transitional basophiles are probably present at earlier stages. The number of basophiles and acidophiles increases between the 10th and 30th days after hatching. During this interval, however, the most significant change is the increase in size and number of distinctive basophiles. The number of basophiles continues to increase up to at least the time of onset of sexual maturity.

Attempts have been made to correlate gonadotrophic activity with the

differentiation of the basophiles. By assaying the potency of the anterior pituitary of chicks at various ages after hatching, Breneman (1945) showed the presence of gonadotrophic activity as early as the fifth day after hatching followed by a sharp rise at 23 days, after which it gradually increases to a still higher level at about 90 days (sexually mature White Leghorn ♂♂). Paralleling these changes, at least from the tenth day on, the basophiles gradually increase in number and apparent secretory activity, whereas the number of acidophiles increases relatively more slowly (Payne, 1946).

Although such a corresponding relation probably has significance in understanding the ontogeny of functional activity of the hypophysis as an integrated glandular organ, care must be taken in assigning a specific hormonal secretion to a particular cell type. The shift in proportional numbers of the three kinds of cells and their changes in cell structure, when correlated with experimental modifications of the interfunctional relations of hypophysis and gonad, comprise a much more reliable index of the cell strain involved. Based on many such studies on immature and adult mammals, the accumulated evidence is consistent, in general, with the view that the basophiles are mainly associated with the production of gonadotrophins. For example, after castration in numerous species of animals (chiefly mammals), cellular changes regularly occur in the anterior lobe. The basophiles increase in number, size, and granular content, while the acidophiles may be much less numerous. Such changes may be repaired after their onset or prevented before their onset by the administration of sex hormones. Moreover, the increased basophilia of the castrate hypophysis is associated with an increase in gonadotrophic activity, as is revealed by functional tests of implants of the castrate hypophysis. On the other hand, at least in some species and under certain altered physiological conditions, the acidophiles may likewise undergo changes such as decrease in number and size and in granular content. By correlating the cellular changes which take place in response to physiological disturbances as a consequence of castration, administration of sex hormones, *etc.*, the hypothesis has been proposed that the basophiles and acidophiles are the respective sites of production of the follicular stimulating hormone (F.S.H.) and the luteinizing hormone (L.H.). It seems not too unlikely that both chromophiles are concerned, perhaps differentially, as has been proposed.

In a similar way, by disturbing experimentally the interfunctional relation of anterior pituitary and gonad (including sex structures dependent upon gonad secretions) during ontogeny, significant new clues may be obtained as to the possible relationship of the time order of differentiation and relative frequency of the cell types at a given stage with the onset and increase of gonadotrophic activity. Specifically indicated is a study of the effects, either separately or in combination, of embryonic castration and the injection of synthetic sex-hormones, each of which, during postnatal stages in mammals, is known to alter the anterior pituitary, both in cytological structure and in the amount of gonadotrophic activity. Also, in ascertaining the possible secretory significance of the granules of the chromo-

philes, cytochemical methods may be useful. For instance, the periodic acid-Schiff reaction indicates that the granules of the basophiles are mucoprotein in nature (Herlant, 1949) and therefore allied chemically to gonadotrophic hormones which contain a proportion of polysaccharides and glucosamine (Li and Evans, 1948).

Such studies as are proposed above might equally well be suitable for ascertaining the time in the course of ontogeny that gonad secretions have a reciprocal action on the anterior pituitary. Although the effects of castration on chicks after hatching and, especially, the effects of the injection of sex-hormones on sex structures of the embryo and of hatched chicks have been widely explored, little or no attention has been directed in such altered physiological conditions to the time of the initial occurrence of changes in the anterior lobe. Sex-hormone activity of the gonad is present early in embryonic development and probably gradually increases with advance in age, especially after hatching. On the other hand, although there is presumptive evidence for the presence of gonadotrophic activity during the latter third of embryonic life, it is not shown to be present with certainty before the fifth day after hatching. From this time on to sexual maturity, gonadotrophic activity gradually attains a higher value. So far as can be judged from the available evidence on mammals, the interlocking of functional activity apparently begins at about the time the gonads are approaching sexual maturity (Engle, 1931). At approximately that time, the gonad secretions stimulate an increased liberation of the gonadotrophic hormones. If correctly evaluated, does this signify that reciprocal functional relations are established when the gonad attains a level of secretory activity sufficient to cause the release of the previously accumulated gonadotrophins? Or is the relation unidirectional at first, during which time the gonadotrophins are only released in sufficient quantities to maintain gonad growth, maturation, and secretory activity? If true, what regulates the gradual release? Answers to these questions await further investigation.

Conclusion. It is hoped that this brief account of accomplishments and reflections on problems of functional development of the avian gonad may have indicated the general lines on which research is progressing and the likely paths of future thought and discovery. New ideas are needed and new physiological methods must be sought to enable us to elucidate such baffling problems as the mechanism of interaction of sex-specific tissue components of the gonad primordia, the tissue site and mode of synthesis of the sex-hormones from precursor chemical substances, the selectivity in response of terminal sex structures to the action of sex-hormones, and the factors concerned in regulating the release of hormones into the circulation in precise quantities in accordance with the requirements of the organism as it grows and develops. Any unexpected discovery along these or other lines may furnish a challenge to any preconceived ideas set forth in this paper.

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THE GENETIC CONTROL OF NORMAL DEVELOPMENT IN THE CHICKEN EMBRYO

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It is important that we should not deceive ourselves. Our knowledge concerning the hereditary forces governing normal embryonic development, of the chick or any other vertebrate, is practically nil. This lack of information about the primary physiological function of genes in normal development should, of course, be a spur rather than a hindrance to future efforts. Fortunately, we are in the possession of hints at least as to the nature and direction such investigations must take if we are to make intelligent progress. I shall, therefore, review briefly the fragmentary evidence relating to the nature of genetic factors in normal development of the chicken embryo and shall indicate some of the paths which future studies may profitably take.

The great differences in adult size of various breeds of fowl have since long excited the curiosity of investigators. It is well known that the most striking dissimilarities in body size of newly-hatched chicks, such as those existing between Bantams and larger breeds, are related to egg size, although it may be doubted that limitations of nutrient supplies and other factors determined by egg size provide complete explanations.

Gregory, Asmundson and Goss (1936) studied the glutathione concentration in embryos of Barred Plymouth Rock and White Leghorn fowl between 5 and 19 days of incubation. Neither egg size nor embryo weight differed significantly in the samples used from the two breeds. In the stocks from which the embryo material came, adult body weight of the Rock females exceeded that of the Leghorn females by 50 per cent and the Rock males were, on the average, 46 per cent heavier than the Leghorn males. Glutathione concentration was consistently higher in the Barred Rock embryos than in those of White Leghorn origin, and the differences for the whole period under observation were highly significant. It would seem, therefore, that in this material the multifactorial genetic differences in post-hatching growth rate and ultimate body size were foreshadowed in the embryonic glutathione values.

Perhaps still more interesting for the present discussion are similar observations on embryos of Cornish and Creeper fowl (Gregory, Asmundson, Goss and Landauer 1939). Two principal conclusions emerged from these studies. One was that Cornish embryos which are homozygous for a micromelia-producing lethal gene (Landauer 1935) had lower glutathione concentrations than their phenotypically normal sibs throughout the period of observation (14-19 days). This deficiency in glutathione may not only play a role in keeping body weight of the lethal embryos below normal, it may also contribute to the differential effect on growth rate of certain body parts (micromelia), and it may, directly or indirectly, be responsible for the inviability of these embryos. The situation is likely to be an expression of the vital importance which the proper genetic control of glutathione production, within given limits, has for normal development.

The second, if tentative, conclusion is presumably even more to the point of our discussion. The data of this study suggested, although they did not prove critically, that distinct racial or breed "types" of embryonic glutathione activity may exist. This is intimated by the following observations. It was found that those embryos from Cornish stock which were homozygous for the recessive lethal and the normal ones gave separate but parallel curves of glutathione content. Another pair of parallel curves, but of a different trend, was found for heterozygous and normal embryos from Creeper stock. Yet another set characterized the embryo material of Barred Plymouth Rock and White Leghorn origin. Sources of error admittedly existed, so that final conclusions must await new determinations under carefully controlled conditions. There seems to be a real opportunity, however, to learn from biochemical observations of this kind something about the physiological nature of genetically-controlled breed differences in growth and development. In a more general way, it may be anticipated that much can be gained from a comparative study of well-defined chemical activity patterns characteristic for developmental stages of genetically distinct stocks.

From the chemical approach, let us turn to embryological investigations. Saunders (1948) has demonstrated in beautiful experiments that the segments of the wing of chicken embryos make their appearance in proximo-distal order, and that a cap of ectodermal tissue which covers the tip of the wing bud plays a decisive role in determining the fate of the wing primordium. After early removal of this ectodermal structure no wing formation whatever will occur. Operation in successively later stages is followed by increasingly more extensive differentiation of the proximal wing parts and a lessening defect in the distal sections. Zwillling (1949) has shown that nature has performed a corroborative genetic experiment in the "wingless" mutation. In homozygotes of this mutant, the apical ectodermal ridge of the wing bud fails to appear and wing formation does not proceed beyond the initial stages. Similar but less drastic defects of the hind limbs of genetically "wingless" embryos arise in the presence of less severe and later-occurring damage to the apical ectodermal ridges of their primordia. It can not be concluded, of course, that in normal development a single gene is responsible for the appearance of the ectodermal ridge tissue and for its subsequent role in differentiation of the extremities, but with such material one may hope to secure further insight into the genetic mechanisms of normal development.

In all such investigations, one must keep in mind that the hereditary agencies which determine and regulate any particular developmental process must be expected to reveal a vastly more complex and intricate situation than is suggested by the effects of single gene mutations. The evolutionary reasons for this are sufficiently obvious, although in practice they often seem to be forgotten. An example of the kind of situation that may be expected was furnished by our studies on the genetics of rumplessness (Dunn and Landauer 1934, 1936; Landauer 1945). For two, apparently unrelated, rumpless mutations, one dominant and the other recessive, multiple modi-

fiers exist, in the presence of which one finds all stages between entire absence of the tail skeleton and its normal development. The manner in which these modifiers made their appearance in our stocks strongly suggested that they were part of a complex genetic system which enhances the stabilization of development in normal stocks. This conclusion was fortified by unrelated observations of two kinds. Zwilling (1942) found that a process of cellular degeneration which occurs in the presumptive tail tissue of normal embryos and which seems in them to serve as a mechanism of tail delimitation is much exaggerated in dominant rumpless embryos, thereby accounting for subsequent lack of the tail skeleton. In the presence of modifying genes, these degenerative processes are restricted to more nearly normal proportions and this may be their actual function in normal stocks. Another indication concerning the nature of the genetic modifiers of rump development was found after the injection of insulin into eggs from several genetically normal-tailed strains of fowl. It appears from this evidence that embryos from stocks carrying plus modifiers of normal skeletal development enjoy greater protection against insulin-induced defects of the axial as well as the appendicular skeleton than do embryos devoid of such modifiers. Again, there is a likelihood that these so-called "modifiers" may be important constituents of the normal genotype. They are, at any rate, the type of small building blocks which were presumably assembled during evolution in order to safe-guard and reinforce, if not actually to direct, vital developmental processes.

Although observations of this kind may shed some light on the nature of the genes controlling normal development, it is indirect light at best, and it is doubtful if by it primary gene events can ever be brought into sharp focus. In order to be of real promise, such investigations must combine the methods of the geneticist, the embryologist and the biochemist. Some years ago we launched a program of this type. In briefest outline, our plan was and still is as follows. The effect of various chemicals on development of the chicken embryo is being tested in order to find substances producing phenocopies, *i.e.*, non-hereditary abnormalities which resemble mutant traits. The physiological nature of such phenocopies and their origin are then studied as adequately as possible. Attempts are made to counteract the teratogenic consequences of phenocopy-inducing chemicals by treatment with supplementary compounds. As a final step, those substances which are effective in protecting embryos against the occurrence of malformations of a phenocopy nature, are tested on the copied mutant stocks in order to determine if a similarly beneficial result, *i.e.*, one modifying the mutant trait toward normal, can be observed.

We have succeeded in the production of phenocopies, at least as judged by gross morphological features, and we were largely successful in finding means of protecting embryos against the action of certain teratogenic substances which induce malformations of a phenocopy type. However, so far as tests have been made to date on mutants, the compounds which, in normal stocks, protected embryos against the occurrence of corresponding phenocopies, proved to be without effect. We have undoubtedly only

scratched the surface of a vast subject and the future may bring more positive results. It is also possible, however, that such experiments cannot succeed, either because the mutant genes change the events of development too early to be repaired by supplementation or because they affect metabolic links different from those involved in the origin of our phenocopies, or because of still other reasons.

Such experiments have one great advantage over many others. Let me illustrate this by reference to radiation techniques which have intrigued both embryologists and geneticists in recent years. Among geneticists it was, and perhaps still is, widely believed that these methods offer much promise as tools for the investigation of elementary biological units. Yet, in criticism of this view, it has been said with good reason that "one would not expect to get a deep insight into the nature of a ship by studying the efficiency of different types of gunfire in sinking it" (Pontecorvo 1951). The same skepticism should be applied to the hope that studies on the developmental effects of major mutant genes will lead to an understanding of the role in growth and development of those genes which make up the normal genotype of fowl or any other higher organism. The damage done by a mutation may, and often does, find expression in the abolishment of a particular and well-defined metabolic function. It does not follow, though this assumption is commonly made, that the normal allele is the sole or even the major factor in this function. If, on the other hand, we succeed in alleviating or repairing damage caused by mutant genes, we may in time secure valuable information about the nature of gene functions in normal development. Studies on the production of phenocopies, together with the search for means by which the organism can be protected against such experimental modification, may offer a technique with which the holes made by our experimental gunfire may be repaired or their occurrence be prevented. This approach should in time provide a great deal of valuable information concerning the developmental physiology of normal embryos, and much may be learned about normal genetic controls if such experiments are made on a comparative basis, using stocks with differing and known genotypes.

The facts presented here, and a few similar ones to which reference could be made, are not new. Obviously, we would be taking the shadow for the substance if any of these observations were hailed as evidence of the manner in which genes direct normal development. Admitting our total ignorance about these events, my only endeavor was to indicate the direction of some paths which may lead toward the desired goal.

The greatest obstacles to rapid progress do not at present lie in the intrinsic difficulties of the problems, although these are great enough, but in failures of organization. Genetic studies on fowl are almost exclusively carried on at the Agricultural Experiment Stations where projects without immediate economic returns find only lukewarm and hesitating support at best. Work on experimental embryology, on the other hand, is rarely undertaken at these institutions and for that reason generally cannot take advantage of genetic breeding techniques. To a large extent, both of these divorced

groups of investigators have lacked the co-operation of biochemists. Means must be found in the future to launch integrated projects in which investigators from all three fields join techniques, imagination and ingenuity for an attack on the basic problems of normal developmental genetics.

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SENSITIVE PERIODS DURING DEVELOPMENT

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Students of embryology cannot help but be impressed with the precise timing of the sequence of events in development. Not only are the origin and onset of function of each organ correlated with those of other organs, but each organ in itself is the result of a precise pattern of interrelated events. Thus, to take a familiar example, the chordal mesoderm exerts a contact effect upon the ectoderm during the time that the latter is capable of reacting to the stimulus. Neither in itself will permit the next step in development unless properly related in time and in space with the other. These problems are, of course, part of the general problem of organization. It is not surprising, therefore, to find that there are certain sensitive periods when the embryo is susceptible to disturbances, either of inherent or external origin. It is our purpose to review some of the better-known periods and summarize some of the known factors which contribute to the sensitivity.

General Sensitive Periods during Normal Development

Our principal concern here will be with the periods of general sensitivity which are revealed when large numbers of eggs from healthy flocks are incubated in the usual commercial incubators. Deaths occur in some of the embryos throughout the entire developmental period, but these are concentrated at two well-defined peaks, one at three to four days and the other at 19–20 days (Payne, 1919; Byerly, Titus, and Ellis, 1933). About three to four per cent of the embryos die at the first such "critical period," and four per cent (Byerly, Titus, and Ellis) to fifteen per cent (Romanoff, 1931) or more at the second. The daily rate of mortality is less than one per cent for the remainder of the period of incubation. Similar peaks of mortality, or critical periods, have been found in other domesticated and wild birds (e.g., Ring-necked pheasant, quail, turkey, pigeon, ring dove, and other wild doves) (Romanoff, 1934; Martin and Insko, 1935; Riddle, 1930), and, if the curves of mortality for each species are redrawn so as to remove the differences caused by different periods of incubation, it is found that the critical periods occur at comparable stages in their development (see Needham, 1942, p. 64). It appears, therefore, that similar factors are operating in each species to make the embryos particularly sensitive at the two intervals of greatest mortality. Some of the factors are well known; others are probable. In the discussion below, we shall consider principally the environmental factors which operate both normally and experimentally to affect the general sensitivity of embryos as indicated by the peaks of mortality. Where it is appropriate, we shall also discuss specific sensitivities which appear at certain times in individual organ systems.

Sensitivity to Physical Conditions

The temperature of incubation is the most obvious physical factor affecting development, since there are upper and lower limits beyond which no

development will take place. The physiological zero, *i.e.*, the temperature below which the blastoderm will not develop at all, is about 27°C. At this temperature, the blastoderm increases in diameter but forms neither vascular tissue nor embryo (Funk and Biellier, 1944). The upper limit at which the embryo will develop is about 41°C., beyond which the cells are killed. For practical purposes, the tolerable range is much more restricted, since hatching does not occur above 39.5°C. or below 35.5°C. when the humidity is kept constant at sixty per cent (Romanoff, Smith, and Sullivan, 1938). The optimal conditions for development seem to be a temperature of 37.8°C. (100°F.) and a relative humidity of sixty-one per cent (Barott, 1937).

Temperatures above or below the optimum accelerate or retard the rate of development, respectively, up to about 10–12 days; from this period onward, variations of the temperature in either direction are growth-retarding. Apparently, the embryo gradually develops its own heat-regulating mechanism from about the sixth to twelfth days, so that high temperatures are more harmful toward the latter part of incubation (*cf.* Henderson and Brody, 1927; Romanoff, 1939, 1941). Abnormalities are common, especially at high temperatures; it is reported that ninety per cent of the embryos which develop at 39.4°C. or above have malformations of the central nervous system (Alsop, 1919). Also, the size and weight of the embryos are markedly reduced by temperatures above 38.5°C. and below 35.5°C. (Romanoff, Smith, and Sullivan). This is attributed to faulty metabolism, since, at both extremes of temperature the assimilation of yolk is slowed, the absorption of the albumen is incomplete, and the amount of allantoic fluid is greatly reduced (*cf.* Barott, 1937, and Romanoff, 1943).

It is not surprising, therefore, to find that changes in temperature have an important effect on the sensitivity of the embryo, and, hence, on the placing of the critical periods. The amount of mortality is heavily increased at both periods, but especially at the later one (19–20 days). This latter is shifted about one day earlier by high temperatures and delayed about four days by low temperature. At the extremes of 33.5°C. and 40.5°C., all embryos are dead at fourteen days (Romanoff, Smith, and Sullivan), a time which is identical with a third critical period in embryos produced by hens on deficient diets (see below). In general, the embryos are much more sensitive to a unit rise in temperature than to an equivalent decrease below normal, as is shown by the comparative mortalities (33.9 per cent at 36.5°C.; 31.7 per cent at 37.5°C.; 72.7 per cent at 38.5°C.; data of Romanoff, Smith, and Sullivan), a fact which indicates that the chick is developing near the upper limit of tolerance of its cells to temperature (Landauer, 1937).

Closely allied to the action of temperature is that of humidity, since the effects of one may be modified by a change in the other. In general, there seems to be an inverse relation between humidity and temperature, within limits, so that when one is increased, the other must be decreased in order to maintain optimal conditions for development (Landauer, 1937). The optimal humidity, as given above, is sixty-one per cent at 37.8°C., or, as usually stated and used, sixty per cent at 38°C.

If the humidity is too low, the egg loses much water, the growth of the embryo is retarded, and it does not obtain as much calcium from the shell, but there is only a small increase in total mortality. When the humidity is too high, the embryo attains a somewhat greater weight and higher calcium content than normal, but the total mortality is over eighty-four per cent (Romanoff, 1930). The increase in mortality at either extreme occurs almost exclusively during the last critical period, at the time of hatching. At low humidity, the embryo may pip the shell but lack the stamina to break its way out. With too much humidity, the chorio-allantoic membrane fails to dry properly so that the embryo literally drowns in its own fluids. The contents of the yolk sac remain very watery, and the sac itself is often not properly withdrawn into the body or is ruptured by the movements of the chick (Romanoff, 1930). These complications of hatching no doubt account for a major share of the mortality at the terminal critical period even under optimal conditions, but they are accentuated by abnormal humidity.

A third physical factor which affects mortality during the critical periods is gravity. It may act in two ways: (1) by rotating the embryo within the shell whenever the egg is turned, and (2) by influencing the position of the embryo at the time of hatching, depending on how the egg was oriented during its incubation. The first of these effects, *viz.*, the rotation of the yolk and embryo whenever the egg is turned, is claimed to prevent the embryo from adhering to the shell before the chorio-allantois is established, and thus would act to reduce embryonic mortality at the first critical period. Experimental embryologists, on the other hand, are often skeptical of the benefits of turning eggs regularly, perhaps excusably so, because they note a worse mortality of their operated embryos when turned than when left alone. However, several independent studies on normal eggs are consistent in reporting a small, but presumably significant, improvement in the hatchability of eggs when turned four or five times a day (*cf.* Landauer, 1937). It would seem, in this case, that the pragmatic approach is best for all concerned.

Gravity is thought to enter into the orientation of the embryo within the egg, especially when the egg is incubated in some position other than horizontal. Byerly and Olsen (1931) report that when eggs are incubated with the small end upward, there is an increase in the number of embryos with their heads located at the small end. Such embryos are at an obvious disadvantage at the time of hatching, since the beak cannot enter the air sac to get extra oxygen, and the head is cramped for space in which to pick through the shell; consequently these embryos usually die. The normal position, with the head located at the large end, is attained between the ninth to twelfth days and becomes fixed during this period because of the increasing size and weight of the embryo (Kuo, 1932). Thus, malpositions of the embryo, whether caused by gravity or other factors, arise during the early part of development, but exert their effect on mortality during the final critical period when the embryo faces the crisis of hatching (*cf.* Hutt, 1929).

The application of gravity in a less direct but more insistent manner, namely, by shaking or jarring the eggs, has led to two results of interest. If eggs are shaken for one to three hours in a test-tube shaking machine immediately before being incubated, there is a marked increase in the number of rumpless embryos and in the incidence of other anomalies (Landauer and Baumann, 1943). Also, if the incubator is jarred at intervals from the fourth to twelfth hours of incubation, sixty per cent of the embryos die between the second and third days, and practically none of them hatch (Stiles and Watterson, 1937). In this latter case, the sensitive part of the embryo is the vascular system, for vitelline vessels fail to organize out of the blood islands. The force is exerted during the highly sensitive period of gastrulation, but its effect on mortality is delayed until the time of the first critical period.

Other physical factors which may influence the sensitivity of the embryo include: irradiation, (which is to be described elsewhere); the time of day at which the egg is laid (Hutt and Pilkey, 1930; McNally and Byerly, 1936); the season of the year (Landauer, 1943); and the age of the laying hen (Landauer, 1937). Except for the first of these factors (irradiation), the nature of the forces involved and their mode of action on the embryo are obscure.

Sensitivity to Chemical Agents

The composition of the air is one of the most obviously important variables in the chemical environment which might influence the viability of the embryo. At the optimal temperature of 37.8°C. and humidity of sixty-one per cent, the most favorable atmosphere consists of twenty-one per cent oxygen and less than 0.5 per cent carbon dioxide (Barott). An increase in the amount of oxygen during early development, especially during the first half day of incubation, is very detrimental, but not so after the second day (Riddle, 1923). In fact, after this initial period of sensitivity, the metabolism of the embryo, as measured by gaseous exchange and heat elimination, is practically the same at percentages of oxygen from 21 per cent to 40 per cent (Barott); beyond these limits it is inhibited. Carbon dioxide in a concentration of 0.4 per cent is beneficial, and perhaps even stimulating, to growth and metabolism during the first week, but 1.0 per cent or more of carbon dioxide results in slower growth and metabolism, many abnormalities, and early death. The maximum combined amounts of the two gases which will permit growth are 22.0 per cent carbon dioxide and 16.3 per cent oxygen (Romanoff, 1930; Romanoff and Romanoff, 1933).

As might be expected, the sensitivity to changes in the optimal proportions of these gases is reflected in the number of deaths at the two major critical periods. The initial sensitivity to excess oxygen may contribute to deaths at the first period; the adverse effects of excess carbon dioxide undoubtedly increase mortality at the last period, for the occurrence of many malpositions (*e.g.*, leg twisted over neck) indicates a struggle against the offending environment (Romanoff and Romanoff). A point of interest in connection with the response of embryos to abnormal concentrations of

oxygen and carbon dioxide is the daily variability in the weights of individual embryos. As is the case with all biological material, there is much variation in the weights of embryos at corresponding times of incubation, but the variation is more extreme at three times: the fifth, thirteenth, and nineteenth days. The first and last periods of greatest variability correspond fairly well with the two periods of greatest mortality. The variability at thirteen days is indicative of another critical period, even though it is not accompanied by any increase in mortality. It has been noted already that extremes in the temperature of incubation result in the death of all embryos at fourteen days, and further evidence to be presented below confirms that there is, indeed, such a critical period at 13-14 days under special conditions.

The middle critical period is best demonstrated in eggs from hens which have been placed on a deficient diet. When the deficiency is of riboflavin, seven-eighths of the resulting embryos die before hatching (Romanoff and Bauernfeind, 1942). The highest mortality now occurs at three to four days (13-14 per cent). Another peak is at 13-14 days (10-11 per cent), and the last critical period, at 19-20 days, is somewhat lower (7-8 per cent). These peaks of mortality are well defined; they rise from a base-line of about one to two per cent mortality, and each covers a period of about three to five days in rising to its height and tapering off. The embryos are dwarfed at all three periods. Those which die at the first period show abnormalities in the vascular area, but it is not clear whether these are the direct cause of death or a result of less obvious disturbances elsewhere. At the mid-period, the affected embryos have many abnormalities in skeletal development, the most common being prognathism, micromelia, and chondrodystrophy. The final critical period is of less importance because much of its usual mortality is absorbed by the earlier ones; those embryos which succumb just before hatching show micromelia and many instances of non-retracted yolk sacs.

Somewhat similar results were reported previously on embryos from hens which were fed on a protein-deficient diet (Byerly, Titus, Ellis, and Landauer, 1935). In this case, the mid-point of highest mortality fell somewhat earlier (11-12 days). The high incidence of skeletal defects, such as micromelia and chondrodystrophy, suggests that these, in themselves, are not specifically caused by a particular kind of nutritional deficiency.

The variety of factors discussed above may now be summarized with respect to their effects on the periods of general sensitivity. The mortality at the first critical period is affected by such diverse factors as high and low oxygen tension, excess carbon dioxide, high and low temperature, mechanical jarring, retention of the egg in the oviduct, *etc.* Certain of these no doubt disturb gastrulation and lead to various malformations which result in the death of the embryo during the early part of the first critical period (two to three days); others interfere with the proper development of the area vasculosa, or lead to degenerative changes in the circulatory network after it is established. Still later (four to five days) the mortality may be due to respiratory difficulties resulting from a poorly-formed allantois. This period is one of rapid organogenesis, so that maladjustments in other organs doubtless take their toll.

The middle critical period is revealed by such different factors as abnormal concentrations of oxygen and carbon dioxide and by deficiencies in the nutritional factors within the egg produced by poor maternal diet.

The last critical period is concerned mainly with the perils of hatching, and includes mortality of such diverse causes as too much humidity (resulting in the drowning of the embryo in its own fluids), abnormal position within the egg (resulting either in asphyxiation, or inability to break out of the shell), non-withdrawal or accidental rupture of the yolk sac, too thick and hard a shell, weakness of the embryo (of many causes), *etc.*

Not only are the causes of mortality diverse for each period, but so also are the times at which the factors act. Thus, at the third critical period, the difficulties of a ruptured yolk sac might not occur until the nineteenth or twentieth day as the chick wriggled within its shell. Other factors, such as too much humidity, are gradual in their effects during the whole of the incubation. Still others may be present, and perhaps active, during early development but with no effect on mortality until the terminal period, *e.g.*, injections of two cc. of 1/20 M. lithium chloride into the egg on the sixth day do not increase the death rate before the nineteenth day (Landauer, 1929).

It is clear, therefore, that each period of general sensitivity is, in reality, a catch-all for mortalities produced by a variety of factors at a variety of times. It is also obvious from the above considerations that each period of general sensitivity is the expression of a number of specific sensitivities which may arise in different organs at different times but coincide in their effects.

We may now consider briefly a few outstanding examples of specific sensitive periods during development:

Such specific sensitivities are involved in the action of a number of chemical agents, including those which arise within the embryo itself, *e.g.*, hormones, and those which may be applied from without. Our mention of them will be confined to general remarks on their relation to tissue sensitivity, since their effects are to be considered in more detail elsewhere in this monograph.

The well-known action of the female sex hormone on the structure and pigmentation of the feathers of Brown Leghorn fowl (Juhn, Faulkner, and Gustavson, 1931) is a prime example of how tissues undergo changes in sensitivity during development, in this case, well beyond the time of hatching. In young chicks, there are no significant differences between the sexes in the structure of their down or of their first juvenile feathers. This remains true under the influence of hormones which arise during normal development and also when massive amounts of female hormone are administered experimentally during early development. However, after producing a variable number of generations of juvenile feathers, and at a time that varies from tract to tract, the feather follicle becomes responsive to the hormone and produces an adult type of feather of altered morphology. Similar considerations hold true for the pigmentation of the feather, either under the influence of the female sex hormone or thyroxin (Trinkaus, 1948, 1950). Differences of this kind are clearly an expression of differences in

the degree of "maturity" or competence of the peripheral tissues (*cf.* Stresemann, 1940). Thus, the circulating hormone, though present long beforehand, cannot exert any action until the tissues become sensitive or reactive. Still other examples of changes in the sensitivity of tissues to hormones are provided by the findings of Roberts, Karnofsky, and Frankel (1951) that cortisone does not act on the chick embryo prior to eight days, and by the report of Zwilling (1951) that insulin produces qualitatively different anomalies depending upon the stage of the embryo into which it is injected.

The chick has been subjected at various stages in its development to the effects of a host of other chemicals with little in common except their toxicity. This factor in itself, however, reveals that certain structures and organs are particularly susceptible or sensitive at certain periods. Thus, solutions of KCN, NH_4OH , NaOH, janus green, methylene blue, and many other depressors or indicators of metabolic activity all act on, or become localized at, the most physiologically active portions of the embryo at the moment. These are: the primitive knot, the posterior end of the streak, the head-process, and the neural folds, at early stages; the brain, venous end of the heart, and all flexures and folds at somewhat later stages; and the points of origin of sensory placodes, visceral furrows, limb and tail buds, and feather germs at still later stages (*cf.* Hyman, 1927; Rulon, 1935). More recently, Spratt (1950) has made a careful study of the action of specific inhibitors of enzymes, and reports that the regions most affected are those which are undergoing the most rapid differentiation.

Every structure or organ of the embryo has its metabolic processes which must be maintained at a certain level for its continued existence, and thus it may be considered to be sensitive, in a broad sense, to any environmental interference with these processes. The sensitivity will, of course, be quantitatively higher during those periods when the organ is most highly charged with metabolic activity. Thus, each organ has its own specific sensitive periods, and these are most likely to fall at the times of its origin, its most rapid differentiation, and its onset of function.

Sensitivity to Contact Stimuli

We may now consider the phenomena of tissue interaction and contact effects (inductions), which are of especial importance during organogeny. Since two tissues are involved, and each must be in the proper state of readiness, or "competence," before the reaction can proceed, it is easy to see why there are specific sensitivities peculiar to these processes.

The formation of the lens under the influence of the optic vesicle is one of the best studied contact effects in the chick embryo. In this case, the inductive tissue is the optic vesicle, and the reactive tissue is the ectoderm covering it. The critical events occur in embryos of 10–20 somites (stages 10–13 of Hamburger and Hamilton, 1951). During this period, the optic vesicle adheres inseparably to the overlying ectoderm; the nuclei of the latter move toward the surface of contact, and become oriented perpendicular to it; and the cells of the future lens elongate into a columnar epithelium, thus becoming more dense (Weiss, 1950; McKeehan, 1951). Im-

mediately following stage 13, the lens ectoderm begins to invaginate, and shortly thereafter becomes less adherent to the optic cup. There is no doubt that the inductive and reactive tissues must be in intimate contact for the effect to take place, for when thin strips of cellophane are inserted between them, the above sequence of events does not take place in areas where there is no contact (McKeehan). The two tissues must also be of the proper age; the optic vesicle remains inductive up to a stage of about 40 somites, but the ectoderm rapidly loses its competence after a stage of four somites (Alexander, 1937).

Thus, there are at least three factors contributing to the sensitivity of the contact effect: the competence of each component tissue, their intimate contact, and the timing of the whole procedure. A disturbance of any one of these would be critical for the entire reaction. Similar considerations apply to other contact effects insofar as they have been studied.

Sensitivity to Infectious Agents

Just as the "competence" of any tissue to participate in contact effects is an indication of its early progress in differentiation, so is the changing sensitivity of its cells to infectious agents a measure of its later progress along the same route. Here may be recorded some observations which, though admittedly preliminary, have a bearing on the problem of when resistance to non-avian diseases arises during the ontogeny of the chick.*

During the course of experiments on the chemotherapeutic effects of p-aminobenzoic acid on typhus infections in chick embryos, it was noted that the drug caused a marked delay in the time of death of the infected embryos. Some of the embryos even lived up to the time of hatching at 19–20 days. This was in striking contrast to the controls, which had received the same infectious dose of rickettsiae at the seventh day of incubation but without p-aminobenzoic acid, for they were all dead within four to seven days after being inoculated (Hamilton, Plotz, and Smadel, 1945). It was found that those embryos which survived to the time of hatching still had a few rickettsiae in their yolk sacs.

An attempt was then made to answer the question as to whether the p-aminobenzoic acid was still exerting an effect 12–13 days after it was administered, or whether the embryo had developed antibodies in the meantime. Samples of blood serum were collected from twenty-two embryos which had survived to the twentieth day, and, in coöperation with the serological section of the laboratory, these were tested for complement-fixing antibodies against both murine and epidemic typhus antigens, along with immune sera of known titer as controls. In no case was there any sign of antibodies in the chick sera in dilutions as low as 1:5 (the lower limit for the test).

In an independent series of experiments on day-old chicks, we had previously found that it was not possible to infect the hatched chicks with typhus rickettsiae. In a typical experiment, an extremely rich inoculum

* The data on which this discussion is based were collected in 1945 while the author was stationed at the Army Medical Research and Graduate School, Army Medical Center, Washington, D. C., as a member of the staff of the Division of Virus and Rickettsial Diseases.

was prepared, consisting of a homogenate of a yolk sac containing countless rickettsiae of the Wilmington strain of murine typhus diluted 1:10 with its own yolk fluid. Injections of this suspension were made both intra-cerebrally (0.2 cc.) and intra-peritoneally (0.4 cc.) into day-old chicks, six receiving the rickettsiae by the first route and four by the second. In no case, did any sign of infection develop. It is clear, therefore, that within a very short period of time the chick changes from a sensitive condition, in which it permits the survival of typhus rickettsiae in its tissues, to an insensitive condition in which it does not. These conclusions are in accord with the findings of Rywosch (1907) and Sherman (1919) that hemolysin for rabbit erythrocytes appears very suddenly in the blood of the chick embryo, and not before the twenty-first day, when the chick is in the actual act of hatching (*cf.* Nace and Schechtman, 1948; Wolfe and Dilks, 1948, for further evidence). It seems probable, therefore, that the change in sensitivity to infectious agents is due to a rather abrupt differentiation of proteins which are either unsuitable for the growth of the micro-organisms or which actively combat them by forming specific antibodies.

General Conclusion

Summing up the above observations, we may conclude, in general, that each organ system has its own specific periods of high sensitivity. These may occur during its formation, during its rapid differentiation, during its onset of function, or in its regulation with respect to the environment or to other systems in the embryo. The disturbance of any system or process may lead to asynchronies which ultimately result in mortality; deaths from such causes may occur at any time during development, but tend to be cumulative at certain general "critical periods," because the normalcy of certain processes, *e.g.*, gastrulation, circulation, respiration, is prerequisite to further development.

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Discussion

DR. DONALD GREIFF (*Saint Louis University School of Medicine, St. Louis, Mo.*): We also have noted the disappearance of rickettsiae at the time of hatching. However, when the yolk sac is withdrawn into the embryo, it is maintained at a higher temperature, and we consider this to be the reason for the disappearance of rickettsiae rather than the differentiation of antibodies.

DR. HAMILTON: This, of course, is a definite possibility in view of the fact that rickettsiae grow more readily at lower temperatures. However, they will also grow, though less abundantly, at higher temperatures during embryonic life, so that I doubt whether such a small change in the temperature of the yolk sac can be the complete answer to the problem of the origin of resistance. Other evidence, more complete than I have been able to present here, indicates that the proteins from which antibodies are formed arise at the time of hatching. Whether they act to change the sensitivity of the embryo to rickettsial infections is not known.

VITAMIN DEFICIENCIES AND ANTAGONISTS

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The effect of maternal diet on embryonic development of the chicken has been the subject of numerous investigations. For the most part, such studies have aimed at determining the quantity of a nutritional factor necessary to support optimum hatchability and subsequent livability of the offspring. Considerable data have been presented which show the effect of a dietary deficiency on time of embryonic mortality and on developmental abnormalities of the embryo. However, such experiments have yielded little information of value in understanding the metabolic processes of the developing chick embryo, even though they are of great practical importance to the poultry industry.

The early application of antimetabolites in the study of biochemical processes, particularly in microorganisms,¹⁻³ proved definitely advantageous, and it appeared logical that a direct approach to the problem of the effect of nutritional factors on embryonic development might be available through the use of such analogues. Moreover, by using antimetabolites, one might ascertain the function of certain nutritional factors in embryonic development which possibly could not be determined by means of maternal dietary manipulation, due to the effect of the deficiency on egg production or to difficulties in designing adequate test rations. In 1948, therefore, experiments were begun at the University of Wisconsin applying vitamin inhibitors to the study of metabolic processes in the developing chick embryo.

The vitamins to be considered in the present discussion are biotin, niacin, vitamin B₆ and folacin. The effect of a maternal deficiency of other vitamins on embryonic development has been reviewed previously.⁴ The experiments with biotin do not involve the use of antimetabolites but will serve to show the effect of inadequate maternal nutrition on embryonic development and to point out the limitations of this approach to the study of biochemical defects in the chick embryo arising from a nutritional deficiency. Only in the case of folacin have there been extensive studies applying the nutritional and antimetabolite procedures to the study of the functions of a vitamin in embryonic development.

Biotin

A maternal deficiency of biotin in the chicken results in a rapid decline in hatchability⁵ and the appearance of extensive gross developmental abnormalities of the embryo.^{6, 7} Parrot beak, crooked tibiotarsus and short twisted tarsometatarsus most frequently occur. In addition, syndactyly, congenital perosis and ataxia have been observed.

A deficiency of biotin results in two peaks of embryonic death, one during the first week of embryonic development and the other during the last three days. As the degree of depletion of the hen increases, the time of embryonic death may gradually shift toward the early period. The most critical

need for this vitamin during embryonic development appears to be during the early stages of growth of the embryo. Injection of adequate biotin into severely deficient eggs prior to incubation negates the deficiency and normal embryonic development ensues. Injections at 120 hours of incubation are of little benefit in promoting normal hatchability. In fact, hatchability declines to zero in an almost linear manner as time of injection is delayed up to 120 hours.

The biochemical defect responsible for death in biotin-deficient embryos is unknown. Of the known functions of biotin, only the fixation of CO_2 in biotin deficient embryos has been studied.⁸ The uptake of radioactive carbonate by normal and deficient embryos was found to be identical. Injections of aspartic acid into biotin deficient eggs also failed to promote embryonic development.

We may logically question the indication of available nutritional data that biotin is most critical during the early embryonic period. Is it not possible that the suboptimal supply of biotin available to the embryo in biotin-deficient eggs is exhausted during early embryonic growth? On the other hand, with a moderately severe deficiency, why do many embryos successfully survive this critical period only to die during the last three days of incubation? Are specific metabolic reactions taking place at these periods which require a greater supply of biotin? Perhaps questions such as these could be answered by the judicious use of analogues of biotin together with metabolites, for which biotin is necessary for synthesis.

Nicotinic Acid

A deficiency of nicotinic acid in the breeding hen has been produced by adding high levels of bone ossein to a purified ration, creating an amino acid imbalance.⁹ With this type of ration, the birds lost weight rapidly and egg production showed a marked drop. Hatchability of the eggs declined to zero by the third week. Nicotinic acid was effective in recovery experiments. Unfortunately, no data were presented dealing with malformations of the developing embryo. It is interesting that such a dietary regime creates a deficiency which will cause reduced hatchability, because nicotinic acid is known to be synthesized during embryonic development.^{10, 11} Clear-cut evidence that nicotinic acid is an important metabolite for embryonic development has been presented through the application of an antimetabolite, 3 acetylpyridine, to a study of the problem.¹² In fact, this was the first paper published applying the antivitamin technique to the study of chemical embryology.

In these experiments, the injection into each egg of 600 micrograms or more of an analogue of nicotinic acid, 3 acetylpyridine, injected into the yolk sac of four-day-embryos was lethal to the embryo, causing death in 24 hours. Sub-lethal doses, or doses rendered sub-lethal by the simultaneous injection of nicotinamide, cause maldevelopment of the embryo. Undersized, deformed legs and a generalized edema at the surface of the body were the malformations observed. Experiments through a wide range of levels of the inhibitor established that the relation between acetylpyridine and nicotin-

amide was fully competitive. The ratio of the inhibitor to the metabolite at which the toxic effects of the inhibitor were nullified was approximately 14:1. Nicotinic acid and tryptophan were found to be far less effective than nicotinamide in reversing the toxicity of acetylpyridine. On the basis of the differential activity of nicotinamide and nicotinic acid or tryptophan in reversing acetylpyridine toxicity, it was suggested that the chick embryo has a mechanism for converting nicotinic acid and tryptophan to nicotinamide at a slow rate. Some direct evidence for the conversion of tryptophan to nicotinic acid in the developing chick embryo has been reported.¹³ The injection of tryptophan into seven-day embryos resulted in an increase in nicotinic acid formed after an additional ten days of incubation. Unfortunately, no other amino acid was tested as a control in this experiment.

Thus it appears that the role of nicotinic acid in embryonic development may be much better determined through the use of antimetabolites than through modification of the dietary regime of the hen.

Vitamin B₆

Nutritional experiments with breeding hens involving vitamin B₆ revealed a critical need for this vitamin by the hen, but no clearcut evidence was obtained to show its importance in embryonic development.¹⁴ The rapid decline in egg production and the development of anorexia precluded a study of this vitamin in the developing embryo. In fact, symptoms of a thiamine deficiency appeared among birds fed a diet deficient in vitamin B₆, presumably because of the limited intake of thiamine as a result of attendant anorexia. For this reason we selected an analogue of vitamin B₆ for our first experiments on the effect of antimetabolites on the developing chick embryo.¹⁵

Our experiments revealed that the injection of 500–1000 micrograms of desoxypyridoxine (2,4 dimethyl-3-hydroxy-5 hydroxymethylpyridine) prior to the start of incubation caused 100 per cent mortality of the embryos. This inhibitory effect of the analogue was largely prevented by the simultaneous injection of 1.0 mg. of pyridoxal hydrochloride. These results demonstrated clearly that vitamin B₆ is highly essential for normal embryonic development. It was observed that most of the embryos died at two to four days of development.

Subsequent experiments revealed that the injection of 1000 micrograms of the analogue at 68 or 72 hours of incubation resulted in an almost immediate death of the embryos whereas this amount was essentially non-toxic when injected at 86 or 92 hours of incubation. At six days of incubation, the lethal dose of desoxypyridoxine was 5.0 mg. and this toxicity could not be reversed by any of the three forms of vitamin B₆. Pyridoxine hydrochloride and pyridoxal hydrochloride also proved toxic at these high levels. Thus the toxicity under these conditions, is not related to the interference with utilization of vitamin B₆ for essential metabolic purposes. At low concentrations of the analogue, such specific interference does occur.

The ratios of vitamin to inhibitor which allowed approximately 50 per cent of the fertile eggs to produce live chicks were for pyridoxal·HCl 1:20,

pyridoxamine dihydrochloride 1:50, and for pyridoxine·HCl 1:100. The metabolic failure in the embryo due to the blocking action of the inhibitor is not known. Neither nicotinic acid nor its amide affected the inhibitory properties of desoxypyridoxine. DL-alanine was also ineffective. The increase in the amount of desoxypyridoxine required to inhibit embryonic development, as the time of injection is delayed, may not be due to any unique function of vitamin B₆ for early embryonic development, but rather, as suggested by the work of Umbreit and Waddell,¹⁶ due to the simultaneous phosphorylation of desoxypyridoxine and pyridoxal, both of which compete for combination with some newly formed apoenzyme with which this co-enzyme functions. If the apoenzyme becomes saturated with pyridoxal phosphate, the desoxypyridoxine phosphate, even if formed, could not then exert its inhibitory action. It may also be that the quantity of the apoenzyme has greatly increased and more of the inhibitor would be required for combination with it to block the reaction which the enzyme catalyzes.

Methoxypyridoxine has been shown to be a more potent antagonist for vitamin B₆ in the chick embryo than is desoxypyridoxine.²⁹ The LD₅₀ of methoxypyridoxine for the embryo was 0.04 mg. per egg at 0 days of incubation, increasing to 0.17 mg. per egg at four days and 3.5 mg. per egg at 13 days. Any of the three forms of vitamin B₆ protected against methoxypyridoxine toxicity. The most active form of the vitamin in preventing the toxicity was pyridoxine, followed in decreasing order by pyridoxal hydrochloride, pyridoxamine and pyridoxal phosphate.

These experiments, while they show that vitamin B₆ is highly essential for early embryonic development, shed no light on the functions of the vitamin in this system. The many known functions of vitamin B₆ in metabolism in other biological systems likely occur in the chick embryo but they remain to be demonstrated. Experiments applying the inhibition technique may aid in determining the functions of vitamin B₆ in embryonic development.

Folacin

The results of nutritional experiments with breeding hens show clearly that folacin is essential for embryonic development.¹⁷ Feeding the breeding hen a diet low in this vitamin results in a marked reduction in egg production, hatchability, and livability of chicks hatched.¹⁸ The deficiency results in two peaks of embryonic mortality, one during the first week of incubation and the second, and more pronounced, at approximately 20 days of development. The injection of folacin into deficient eggs prior to the fourth day of incubation permits normal embryonic development.¹⁹ Injections as late as the 17th day of incubation markedly improve hatchability but do not result in strong, vigorous chicks. In fact, the evidence suggests that sufficient quantities of the vitamin must be available during the greater part of the embryonic period if "normal" chicks are to be hatched.

The reason for the high embryonic mortality at 20 days of development is unknown. No anemia was observed. Grossly, the embryos appear quite normal but die, apparently at the beginning of pulmonary respiration. It seems unlikely that the metabolic failure at this period is the end result of

some earlier failure because of the effectiveness of injecting folacin as late as the 17th day of incubation.

Symptoms of an embryonic folacin deficiency which we observed were skeletal abnormalities of the beak and tibiotarsus, syndactyly, and dwarfism.

The application of an analogue of folacin (aminopterin, 4 aminofolacin) to the study of the function of this vitamin in embryological development has proven quite fruitful. Karnofsky and co-workers²⁰ determined the effect of a number of compounds related to folacin on development of the chick embryo. The LD₅₀ for four-day embryos was determined. The 4 amino folic acids were found to be most inhibitory, the LD₅₀ being approximately three micrograms per egg. This inhibition was not reversed by folacin.

Abnormalities of the embryo due to the inhibitory action of folic acid analogues were described. Embryos dying during the first four days after treatment were severely stunted. Embryos surviving eight to ten days showed the greatest changes. The allantoic vascular system appeared abnormal and the amnion was distended with a clear watery fluid. Developmental abnormalities were evident, the most common being a flattened head, small eyes which frequently had a clear sac over the lens, absent or underdeveloped lower mandible, a long and usually twisted neck, exteriorization of the viscera, abnormal or absent toes and generalized edema. Similar gross abnormalities were observed in our nutritional experiments with folacin.

Our experiments with an analogue of folacin (aminopterin, 4 amino folic acid) were designed to obtain evidence regarding the function of folacin in embryonic development.²¹ Previous work with lactic acid bacteria had implicated folic acid in the synthesis of thymine and purine bases, since these substances will replace folic acid for growth of these organisms,^{22, 23} and further, these substances counteract the growth inhibition of *Lactobacillus casei* by methylfolic acid.²⁴ Thymidine, in the presence of purine bases, counteracts the inhibitory effect of aminopterin on *Leuconostoc citrovorum*.²⁵ In the case of a strain of *Escherichia coli*, thymidine, but not vitamin B₁₂, thymine, hypoxanthine or hypoxanthine desoxyriboside, counteracts aminopterin inhibition.²⁶

With this evidence available regarding the function of folacin in bacterial systems, experiments were undertaken to determine if the same possible mechanisms may function in the animal system.

TABLE 1 shows the effect on hatchability and time of embryonic mortality of injecting eggs with varying quantities of aminopterin prior to incubation. Amounts in excess of 100 micrograms per egg resulted in 100 per cent mortality at 0-1 day of development. Grossly, such eggs could not be distinguished from infertile eggs. The toxic effect of aminopterin declined as embryonic development progressed. Twenty micrograms of the inhibitor was completely inhibitory when injected at 0 or three days of incubation. At three days, death was almost immediate. At six days, a delayed toxicity was observed which frequently produced death two to six days after the injection. At nine or more days of incubation, this amount of the inhibitor was non-toxic. The same possible explanation for the decreased toxicity

of this inhibitor as development progresses may be given as the explanation for the vitamin B₆ analogue, *i.e.*, the apoenzyme becomes saturated with the coenzyme and the inhibitor cannot exert its action, and the apoenzyme increases as the embryo grows, necessitating more inhibitor, simply to exert its action.

The effectiveness of various compounds in counteracting aminopterin inhibition was determined by injecting them with the aminopterin prior to incubation. Folacin, vitamin B₁₂, thymine, hypoxanthine and hypoxanthine desoxyriboside were without effect in reversing the toxicity. Thymidine, however, was effective in prolonging the time of embryonic survival, and permitted a hatch of ten per cent of live chicks. Thymidine in combination with hypoxanthine desoxyriboside was still more effective in delaying time of death and permitted a hatch of 32 per cent of chicks. This

TABLE 1
EFFECT OF AMINOPTERIN ON HATCHABILITY OF EGGS

Substance injected	Amount injected ug.	Number of eggs	Percentage of embryos dying at:			Percent- age of hatch
			0-1 day	2-5 days	6-20 days	
Aminopterin	1000	31	100			
"	500	15	100			
"	200	15	100			
"	100	15	100			
"	50	30	96.7	3.3		
"	20	115	73.9	22.6	3.5	
"	10	15	53.4	33.3	13.3	
"	5	30	36.7	23.3	16.7	23.3
"	2	15	26.7	13.3	6.7	53.3
Water (.1 cc)		129	11.6	6.2	11.6	70.5
None		128	11.7	4.7	2.3	81.3

combination essentially duplicated the results obtained by injecting with the aminopterin an enzymatic digest of desoxyribonucleic acid.

More recent experiments²⁷ testing thymidine in combination with various purine and pyrimidine bases, their ribosides and desoxyribosides indicate that the purine bases hypoxanthine, guanine, and adenine are of value, when injected with thymidine, in counteracting aminopterin inhibition. Thymine and uracil were ineffective. The riboside of hypoxanthine appeared to be somewhat more effective than hypoxanthine itself or the desoxyriboside. It would appear that the chick embryo and *Leuconostoc citrovorum* respond similarly to aminopterin inhibition since, with this organism, the purine bases are required in the media before thymidine will reverse aminopterin inhibition. The synthesis of these compounds is necessary for nucleic acid formation, and the absolute failure of embryonic growth to begin as a result of aminopterin inhibition is consistent with this failure.

A further example of how analogues can be used to study biological mecha-

nisms in the chick embryo is provided by work showing that synthetic folinic acid is effective in counteracting aminopterin inhibition²⁸ under conditions where folacin is ineffective. This study provides additional evidence for the mechanism of action of folacin or its derivatives in embryonic development. From these experiments and those previously cited with bacterial systems and chick embryos, the thesis is developed that folic acid is first converted to the citrovorum factor which functions, either directly or indirectly, in the synthesis of thymidine and one or more of the purine desoxyribosides from unknown precursors.

It is possible that such mechanisms could have been determined by obtaining a sufficient number of folacin-deficient eggs and conducting similar experiments using products for the formation of which folacin is thought to be necessary. Such a procedure, however, would have been much more time-consuming and expensive. It is not necessarily true that the metabolic process first blocked by the inhibitor would be the first affected by a nutritional lack. The two approaches might yield different data. Also it might be possible to study a particular defect or stage of development by one approach and not the other. It is likely that complete nutritional data, together with studies utilizing the inhibitors, will provide a much clearer picture of the functions of the vitamins in embryonic development than either technique alone.

In presenting these cases of application of inhibitors to the study of metabolic reactions in the chick embryo, I do not wish to give the impression that this technique may be used to solve all the problems of function of vitamins in the chick embryo. The data presented show that the antagonists may be used to attack the problem in a new and different manner.

Solubility of compounds prohibits injection of large amounts of some compounds early in incubation, since injections over 0.2 ml. decrease hatchability. The ideal situation in using inhibitors would be one in which the inhibitor blocks a certain reaction and, by supplying the product of the reaction preformed, the blocking effect is overcome. If, on the other hand, a piling up of a substrate due to the blocked reaction caused death of the embryo, no information could be gained in injecting the product of the reaction. In such cases, however, information might be gained regarding the beginning of functioning of various enzyme systems.

The developing chick embryo carries out most of the metabolic reactions of higher animals in an enclosed system. This system is free from some of the complications of nutritional experiments, such as intestinal synthesis and other environmental factors. The chick embryo is readily obtained and easily subjected to various experimental treatments. Experimental data accumulated to date shows how vitamin antagonists may be applied in a study of the metabolic reactions in the chick embryo. It should be pointed out that most of the experiments with the chick embryo to date were analogous to those previously conducted with micro-organisms, and have, for the most part, yielded similar results. This may be taken as an indication of the unity of many metabolic processes in widely diverse organisms. It seems to me that we may look upon the fertilized egg as a

living medium that offers considerable opportunity for research dealing with vitamin functions. I believe the vitamin inhibitors provide us with a very useful tool for using this admittedly complex medium in a study of vitamin function in animal tissues.

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THE EFFECTS OF SOME HORMONES ON DEVELOPMENT

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Most of the studies of the effects of hormones on chick embryos have revealed that the tissues which react to these substances are essentially the same as those which respond in the adult. While the reactions frequently differ in detail, they are, nevertheless, of the same nature as those of the adult tissues.

In at least one case, that of insulin, we have an example of a hormone which produces morphological effects which are unlike any encountered in adults. These are largely the result of alterations of the metabolism of the embryo. The embryonic tissues, which seem to have quite precise requirements during their early development, respond to the altered metabolism in a manner which we shall describe below.

Before going into this discussion, it might be well to give a brief account of the major part of the literature dealing with the more usual reactions of embryonic tissues (see Moore, 1951 for a recent review). The most thorough studies of hormonal effects on chick embryos have been made with the sex hormones (see Willier in this symposium and Snedecor, 1949 for literature). These indicate that the gonads respond to the heterologous sex hormone by changing either to a gonad of the other sex or by acquiring intersexual characteristics. Female hormones cause the Müllerian ducts of males to persist and frequently to hypertrophy, while male hormones cause the Müllerian ducts of genetic females to regress and their Wolffian ducts to hypertrophy. Most of the differences in response between embryo and adult are due to the presence of tissues in the embryo which degenerate or regress by the time the adult condition is achieved. The adult testis, for example, lacking cortical (ovarian) tissue, cannot be transformed to an ovotestis as can the embryonic testis which still has cortical elements.

Certain general reactions of the chick embryo are also similar to those encountered in adult or young animals. Total hypophysectomy (Fugo, 1940) of early embryos causes a dwarfing which is presumably due to the absence of the growth hormones. Thyroid deficiencies, obtained by thiourea or thiouracil treatments (Adams and Bull, 1949) also produce dwarfing. It is interesting, in this regard, to note that excess of thyroid hormone obtained by means of grafts of adult thyroid tissue (Willier, 1924) or brought about by stimulation of the embryonic thyroid gland with thyrotropin (Woodside, 1937) also interfere with growth of the embryo. On the other hand, direct administration of thyroxin does not produce this effect (Hanan, 1928, Beyer, 1951). Adrenal cortical preparations have also been shown (Landaauer, 1947 and Karnofsky *et al.*, 1951) to produce a proportionate dwarfing. It may be well to note that while the dwarfing effects produced by these different substances may be superficially alike, they undoubtedly result from considerably different actions of the various hormones.

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Many of the specific effects of hormones also have been demonstrated in the chick embryo. The gonads respond to gonad stimulating hormones of the pituitary gland (Domm and Dennis, 1935, Domm, 1937) and to pregnancy urine (Breneman, 1935, Venzke, 1941). The embryonic thyroid gland responds to adult thyrotropic preparations (Woodside, 1937) and to the embryonic thyrotropic hormone (Martindale, 1941). This gland also responds to thyroprotein and to KI by becoming goiterous (Wheeler and Hoffman, 1948a, 1948b, 1949). Embryonic skin has been shown to become cornified under the influence of the thyroid hormone (Bartels, 1944). Fugo's hypophysectomy experiment with New Hampshire Red embryos produced a melanotic condition in the down feathers. This effect could be traced to thyroid deficiency, since injection of thyrotropic hormone alleviated it. On the other hand, Trinkaus (1950) could not demonstrate any effect of thyroxin on the down pigmentation of Brown Leghorn embryos.

In like manner, it has been found that many of the physiological responses are similar in the embryo and adult. Adrenaline causes an increase in heart-beat rate (Matsumori, 1929) and a rise in blood sugar (Vladimiroff, 1931). Thyroxin produces a decrease followed by an increase in CO_2 production (Hanan, 1928), a glycogenolysis of embryonic liver glycogen (Dalton and Hanzal, 1940) and an increased O_2 consumption (Beyer, 1951). Adrenal cortical preparations produce a rise in blood sugar (Zwilling, 1948) and a fall in liver glycogen (Dalton and Hanzal, 1940). This hormone also causes a leukocytosis in the embryonic blood (Weller and Schechtman, 1949). Dalton and Hanzal also report that a crude anterior pituitary extract results in a rise in blood sugar and an increase in both skeletal muscle and liver glycogen. These data are scant and incomplete but are representative of the few studies on the physiological effects of hormones on the chick embryo.

As mentioned above, insulin, unlike most of the other hormones studied, has been found to have morphological effects which are unique to the embryo (Landauer, 1945, 1947a). When insulin is injected into the yolk of embryos during the first three days of incubation there is a considerable incidence of caudal anomalies. Tail structures are either completely absent or partly suppressed. If insulin is administered to embryos of four to seven days incubation, one no longer encounters caudal abnormalities. Instead, there is a high incidence of an achondroplastic-like micromelia. With higher doses of insulin this condition is usually accompanied by a dwarfing of the embryos. Beak and eye anomalies may also be encountered.

An investigation into the effects of insulin on the carbohydrate metabolism of chick embryos was undertaken. Previous investigations (Hanan, 1925, Dalton and Hanzal, 1940) had shown that insulin lowers blood sugar in treated embryos. These results were obtained with short term experiments upon relatively old embryos (8, 12 and 14 days) which no longer showed the morphological reactions to insulin. Our data (Zwilling, 1948) revealed that embryos injected with insulin on the fifth day of incubation become hypoglycemic and that the lowered blood sugar following a single injection may persist until the twelfth day of embryonic life. This was found to be the

case more frequently after large doses of the hormone were administered. In every case, though, blood sugar values returned to normal levels by the fourteenth day. When the course of glycemia in single embryos was followed and then related to the morphological reaction of the embryo, we found that embryos which were hypoglycemic for the full period (between the sixth and twelfth days) were also the ones which exhibited the most extreme micromelia. Those which recovered normal sugar levels between the eighth and tenth days had, on the whole, only moderate leg shortening, while those which showed recovery by the eighth day were indistinguishable from normal embryos.

In addition to the above, we found that cortin, which exaggerates (Landauer, 1947b) the micromelic effects of insulin when it is administered at the same time, also accentuates the hypoglycemia. This was most evident in the persistence of the low blood sugar values far beyond the normal recovery period. It seemed, from all of these data, that the micromelia was associated with hypoglycemia in such a way that the more severe micromelias were found in embryos which had the lowest blood sugar values for the longest time.

In 1948 Landauer reported that the morphological effects of insulin could be prevented by simultaneous (and somewhat prior or subsequent) administration of nicotinamide. This was also found to be the case, though to a lesser extent, with α -ketoglutaric acid. This was of great interest to us since all of our attempts to mitigate insulin micromelia with added glucose had failed (unpublished). In fact, the glucose seemed to exaggerate the micromelia. Assays for blood sugar revealed that the nicotinamide also eliminates the hypoglycemia far earlier than if it is not administered (Zwilling, 1949). Some of our unpublished data reveal that the micromelia-alleviating effect of nicotinamide is quite selective. It prevents (or reverses) only the effects on the limbs but does not prevent the decrease in size of the rest of the embryonic body.

These data make it evident that disturbances of the carbohydrate cycle, at some point, are involved in the production of the anomalies by insulin. It is still not clear whether there is a direct antagonism of nicotinamide-requiring compounds or whether the nicotinamide requirement of the long bone cartilage is increased as a result of the diminished carbohydrate supply which is a consequence of the hypoglycemia. Contrary to expectations at least two nicotinamide antagonists, 3-acetyl pyridine and pyridine-3-sulfonic acid, do not produce micromelia in chick embryos (Zwilling and DeBell, 1950). This evidence may argue in favor of an indirect effect of insulin on nicotinamide requirements of the embryo.

Recently (Zwilling, 1951) we have obtained data which suggest that the insulin hypoglycemia of the embryo results from the action of this hormone on the yolk-sac membrane. This tissue reacts to insulin in the same way that adult mammalian liver and muscle does under certain conditions, *i.e.*, its glycogen content increases considerably. All of the carbohydrates in the embryo proper are diminished under the influence of insulin; presumably a consequence of the decreased carbohydrate supply transmitted to the embryo by the yolk-sac. Adrenal cortical hormone (unpublished data)

alone has relatively little effect on the yolk-sac glycogen. When it is combined with insulin, however, the glycogen content of the yolk-sac membrane is increased significantly more than after the action of insulin alone. These results are consistent with our previous observations on the glycemic effects of the cortical hormone.

Thus, we have seen that, although the morphological responses of the chick embryo to insulin are unique, its physiological reactions are quite similar to those which are expected from adult tissues. Despite the fact that we are uncertain about the precise role of nicotinamide in this picture, we feel that there is a considerable likelihood that the increased requirement for this substance is mediated via the other carbohydrate disturbances. Other participants in this symposium have clearly demonstrated that even early embryonic rudiments acquire specific nutritional requirements as well as other specificities. It is, therefore, quite feasible to interpret the morphological responses of the embryos as consequences of the insulin's interference with these specific requirements at critical times in the development of the involved structures.

The literature dealing with experimental teratology is characterized by the frequency with which the same type of abnormality is produced by a variety of seemingly unrelated treatments. Micromelia which is similar to that produced by insulin has likewise resulted from a wide variety of treatments. These are: (1) a dietary deficiency of unknown nature whose effects can be mitigated by wheat germ, liver and whey (Byerly, Titus, Ellis and Landauer, 1935); (2) manganese deficiency (Lyons and Insko, 1937); (3) riboflavin deficiency (Romanoff and Bauernfeind, 1942); (4) sulfanilamide (Ancel, 1945); (5) eserine sulfate (Ancel, 1945); (6) biotin deficiency (Couch, Cravens, Elvehjem and Halpin, 1948); (7) folic acid antagonists (Karnofsky, Patterson and Ridgway, 1949); and (8) thallium salts (Karnofsky, Ridgway and Patterson, 1950).

Several of the above conditions (Mn deficiency and the B vitamin deficiencies) are undoubtedly related to distortions of carbohydrate metabolism. Most of these substances are important components of coenzymes. The information which we have presented above relates insulin to this group, both through its direct effects on carbohydrates and by virtue of the role of nicotinamide in the reversal of the morphological aberrations. Apparently, the effects of two other substances, sulfanilamide and eserine sulfate, are mediated via carbohydrate metabolism disturbances since they too are reversed or eliminated by nicotinamide therapy (Landauer, 1949; Zwilling and DeBell, 1950). Thus we find that all of these seemingly unrelated means of producing micromelia, except treatment with thallium salts, may be related, through one path or another, to aberrant carbohydrate metabolism. Such similar effects of dissimilar substances would have been explained formerly as the result of non-specific depression at a common critical time or stage of development. The revelation that similar metabolic derangements are involved, at least in the case of experimental micromelias in chick embryos, places the emphasis on the critical specific requirements of the reacting tissues as well as on the stage when they are affected.

In summary, insulin produces morphological responses in chick embryos

which are unlike any encountered in adults. This hormone produces metabolic disturbances which are consistent with its action in adults. The unusual reactions to these metabolic disturbances are the result of the dependence of the developing embryonic tissues upon a rather precise set of nutritional and enzymatic conditions. Material is presented which relates the teratogenic action of a number of seemingly unrelated substances to disturbances of carbohydrate metabolism.

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Discussion

DR. PETER GRUENWALD (*State University of New York, New York, N. Y.*): You are probably aware of the fact that teratological conditions which seem to be the same may be the result of entirely different developmental events.

DR. ZWILLING: Yes, I am aware of this. Aside from the superficial similarity in these cases of micromelia in chicks, however, we have the coincidence that several of them are mitigated by nicotinamide, and B vitamins are involved with some of the others. This indicates some similarity in the etiology of these conditions.

DR. GRUENWALD: Have the histological details been studied in these various micromelias?

DR ZWILLING: Not in most of them. Ancel has studied the sulfanilamide-induced micromelia and has classed it as an achondroplasia. Landauer studied Mn-deficient embryos and decided that they were not achondroplastic. To my knowledge, the other have not been studied in detail.

DR. HEINRICH WAELSCH (*New York State Psychiatric Institute, New York, N. Y.*): Have you any indication of why the blood sugar and possibly glycogen return to normal levels by the 14th day?

DR. ZWILLING: We have not been able to follow glycogen of the yolk-membrane later than the 12th day. It is virtually impossible to clear it of adhering yolk in these later stages. The 12th day period seems to be of great importance for the embryo. It is at about this time that many of the embryonic structures achieve definitive function: insulin is secreted,

the liver begins to store glycogen very actively, *etc.* We can only interpret the return to normal at this time as the result of the embryo's own control mechanism coming into play.

QUESTION: Have any other substances in the carbohydrate chain been used and do they have effects like nicotinamide?

DR. ZWILLING: Some others have been tried, mostly by Dr. Landauer. I recall that coenzyme I itself had no such effect. Possibly Dr. Landauer could elaborate more.

DR. LANDAUER: Several substances, such as pyruvic and lactic acids, have very weak mitigating action on insulin-micromelia. The results are different when these substances are used on earlier stages, where rumplessness is involved.

THE EFFECTS OF METALS ON THE CHICK EMBRYO: TOXICITY AND PRODUCTION OF ABNORMALITIES IN DEVELOPMENT*

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The stimulus for a study of the effects of the various elements on the development of the chick embryo was initiated by the striking effect produced by thallium sulfate injected into the yolk sac of the four-day-old embryo.¹ In order to determine the specificity of this effect, 53 other elements were assayed against the chick embryo. The current status of the work is presented in this report.

Materials and Methods

The effects of the elements were studied principally in the four- and eight-day-old embryo since developmental abnormalities are often grossly evident at these older ages. Furthermore, elements generally toxic to embryos early in development may produce selective growth effects when given to more differentiated embryos. Elements found to produce specific and consistent abnormalities when injected at four or eight days were studied further in younger and older embryos.

Commercially supplied White Leghorn eggs were used. They were maintained at a temperature of 38°C, and candled daily for viability. The volume of the test agent injected per egg ranged from .05 cc. to 0.2 cc. with an occasional yolk sac injection of 0.3 or 0.4 cc. When possible, water soluble salts were used, but, in rare cases, insoluble salts were ground in distilled water and injected as a suspension. The injections were made by two routes: into the yolk, or onto the chorioallantoic membrane (CAM) of eight-day eggs. Yolk sac injections were made through a hole drilled in the blunt end of the egg, and the opening then was sealed with melted paraffin. The CAM was injected through a small window cut in the shell directly over the membrane and the opening was sealed with Scotch tape after the injection.

The preliminary toxicity tests were carried out in the four-day-old embryo. Four eggs were injected at each of three of four dose levels, and, after several trials, the LD₅₀ dose was approximated. Groups of six to ten eggs were injected at several dose levels above and below the estimated LD₅₀ in order to obtain a more precise figure. In many instances, several confirmatory toxicity runs were necessary to obtain a reasonably consistent LD₅₀ figure. With the four-day yolk sac data as a guide, similar toxicity studies were completed in the eight-day embryo by the yolk sac and CAM routes.

The embryos were candled daily and, each day, the dead embryos were recorded and examined. Occasionally, embryos were sacrificed for specific

* This work was supported by grants from the National Cancer Institute of the United States Public Health Service, the Damon Runyon Memorial Fund for Cancer Research and the American Cancer Society.

reasons during incubation but, in most instances, toxicity experiments continued until the 18th day of incubation, when all embryos were sacrificed. At this time, the embryos were weighed and examined grossly for abnormalities of the skin, feathers, bone, brain and internal organs. The membranes and yolk were also inspected.

Results

Toxicity data. The LD₅₀ of 54 elements in various forms, when injected into the chick embryo at four and eight days of development, are summarized in TABLE 1. When more than one salt of an element was tested, they are listed with the most toxic salt of that element. There are numerous factors which influence the toxicity of an element in the fertile chicken egg, besides the inherent or direct toxicity of the chemical at its locus of action. These factors are related to:

(a) *The egg.* The egg varies in size and chemical composition, the latter being related to the diet of the hen to a considerable extent. Certain excesses or deficiencies of minerals or vitamins, for example, may modify the response of the embryo. An extremely important factor is the developmental age of the embryo at the time the chemical is injected, since the presence of specific organs or metabolic systems may be critical in determining the effect of a chemical.

(b) *The state and properties of the injected chemical.* The route of injection and the vehicle used may be very important in the rate of absorption of the chemical. Certain elements may react with non-embryo constituents of the egg, may be precipitated at the site of injection, or may be converted by combining with substances in the egg to form a substance of greater physiological activity.

(c) *Distribution and metabolism of the injected chemical.* The rate of absorption and the toxicity of high blood levels of an element are important factors in toxicity. Some chemicals are concentrated in the albumen or in the allantoic sac, and thus detoxified as far as the embryo is concerned. Other chemicals may be deposited in inactive form in some parts of the embryo, as the bones, and thus be rendered less toxic.

All of these factors must be taken into account in interpreting the toxicity or teratogenic effect of an element. In FIGURE 1, the elements have been divided into four groups, based on their toxicity in the four- and eight-day embryo, by the yolk sac and CAM routes. These groupings suggest how some of the factors mentioned above influence the activity of specific chemicals.

Specific Effects on Embryonic Development. Under appropriate circumstances, it is probable that an excess of any element can be shown to have some specific effect on embryonic development. In many instances, however, the element appears to be generally toxic and the embryo either dies promptly or, if the embryo survives, it will develop normally. On the other hand, situations have been found in which some elements will exhibit specific biological activity in the chick embryo. Some elements exert their effect when given in any chemical form, at any time during incubation, and

TABLE 1

TOXICITY OF SALTS OF VARIOUS ELEMENTS IN FOUR- AND EIGHT-DAY CHICK EMBRYOS

Element	Formula of Salt	Estimated LD ₅₀			
		Mgs/egg	Microatoms/egg*		
			4-day	8-day	8-day CAM route
		Yolk sac route			
Arsenic	KAsO ₂	0.02	0.10	0.34	0.07
Arsenic	Na ₂ HAsO ₄ ·7H ₂ O	0.30	0.96	2.60	0.32
Arsenic	As ₂ O ₅	0.16	1.40		
Cadmium	CdCl ₂	0.02	0.13	0.22	0.09
Selenium	H ₂ SeO ₄	0.03	0.17	0.55	0.11
Platinum	PtCl ₄ ·2HCl·6H ₂ O	0.13	0.25	>19.0	0.17
Lead	Pb(NO ₃) ₂	0.10	0.30	4.50	3.00
Mercury	HgCl ₂	0.08	0.30	3.10	0.21
Chromium	Na ₂ Cr ₂ O ₇ ·2H ₂ O	0.06	0.40	0.30	1.02
Chromium	Cr(NO ₃) ₃ ·9H ₂ O	0.80	2.00	>50.0	7.50
Antimony	SbCl ₃	0.10	0.44	2.20	0.08
Silver	AgNO ₃	0.10	0.59	0.50	0.50
Nickel	NiCl ₂ ·6H ₂ O	0.20	0.84	10.0	1.40
Vanadium	VO(CH ₃ COO) ₂	0.20	1.10	0.65	0.24
Tungsten	Na ₂ WO ₄	0.78	2.60	8.50	6.30
Molybdenum	Na ₂ MoO ₄ ·2H ₂ O	0.80	3.30	2.90	14.0
Copper	CuCl ₂ ·2H ₂ O	0.60	3.50	29.0	0.71
Strontium	Sr(NO ₃) ₂	0.80	3.80	>94.0	54.0
Cobalt	CoCl ₂	0.50	3.80	7.70	2.30
Tellurium	Na ₂ TeO ₃	1.00	4.50	18.0	0.12
Osmium	OsO ₄	1.20	4.70	9.90	0.99
Thallium	Tl ₂ SO ₄	1.30	5.20	5.50	2.80
Lanthanum	LaCl ₃ ·7H ₂ O	6.00	16.0	54.0	2.80
Zinc	ZnSO ₄ ·7H ₂ O	5.00	17.0	>69.0	1.20
Cerium	Ce(NO ₃) ₃ ·6H ₂ O	8.00	18.0	>46.0	3.00
Manganese	MnSO ₄ ·4H ₂ O	5.00	21.0	>86.0	1.50
Uranium	UO ₂ (C ₃ H ₃ O ₂) ₂ ·2H ₂ O	15.0	22.0	>29.0	0.44
Indium	InCl ₃	5.00	23.0	>91.0	21.0
Ruthenium	RuBr ₂	7.00	27.0	>76.0	9.50
Iron	FeSO ₄ ·7H ₂ O	8.0	29.0	25.0	2.00
Iron	FePO ₄ ·2H ₂ O	15.0	80.0	75.0	2.90
Thorium	Th(NO ₃) ₄ ·4H ₂ O	20.0	38.0	>38.0	14.0
Fluorine	NaF	1.75	42.0	71.0	30.0
Iridium	IrCl ₃	14.0	47.0	13.0	1.30
Iodine	KI	8.0	48.0	60.0	78.0
Tantalum	K ₂ TaF ₇	20.0	51.0	36.0	6.40
Tin	SnCl ₂	10.0	53.0	>105.0	7.40
Tin	SnCl ₄	20.0	77.0	77.0	5.40
Gallium	GaC ₆ H ₅ O ₇	15.0	58.0	77.0	2.70
Magnesium	MgSO ₄ ·7H ₂ O	15.0	61.0	>121.0	105.0
Lithium	LiSO ₄ ·H ₂ O	8.0	62.0	78.0	64.0
Zircon	ZrOH(C ₃ H ₃ O ₂) ₃	18.0	63.0	>70.0	18.0
Boron	H ₃ BO ₃	5.0	81.0	113.0	81.0
Aluminum	AlCl ₃	15.0	112.0	>150.0	16.0
Silicon	Na ₂ Si ₃ O ₇	20.0	247.0	>247.0	31.0
Germanium	GeI ₂	>10.0	>31.0	31.0	3.10
Gold	AuCl ₃ HCl·4H ₂ O	>20.0	>41.0	>41.0	1.20
Rhodium	RhCl ₃	>10.0	>48.0	72.0	4.30
Bismuth	BiCl ₃	>20.0	>63.0	>63.0	11.0
Yttrium	Y(NO ₃) ₃ ·6H ₂ O	>30.0	>78.0	>52.0	2.90

TABLE 1 (CONTINUED)

Element	Formula of Salt	Estimated LD ₅₀			
		Mgs/egg	Microatoms/egg*		
			4-day	8-day	8-day CAM route
		Yolk sac route			
Barium	BaCl ₂ ·2H ₂ O	>20.0	>82.0	45.0	3.30
Palladium	PdCl ₂ ·2H ₂ O	>20.0	>94.0	>94.0	19.0
Titanium	Ti ₂ (SO ₄) ₃	>20.0	>104.0	89.0	29.0
Beryllium	BeSO ₄ ·4H ₂ O	>20.0	>113.0	>113.0	5.60
Cesium	CsCl	>20.0	>119.0	>119.0	77.0
Rubidium	RbCl	>30.0	>248.0	>248.0	124.0
Calcium	CaCl ₂	>30.0	>270.0	270.0	23.0
Potassium	KCl	>50.0	>671.0	>537.0	134.0
Sodium	NaCl	>50.0	>855.0	>685.0	145.0

* Microatoms/egg = $\frac{\text{LD}_{50} \text{ in mg/egg} \times 1000}{\text{Mol. wt. of salt in gms.}} \times \text{no. atoms of element in salt}$. The LD₅₀ in mg/egg for each salt for the eight-day embryos is calculated easily from the relationship between the mg/egg and microatoms/egg data presented for the four day embryo.

DIAGRAM OF TOXICITY RELATIONSHIPS OF ELEMENTS, AS RELATED TO AGE OF EMBRYO AND ROUTE OF INJECTION

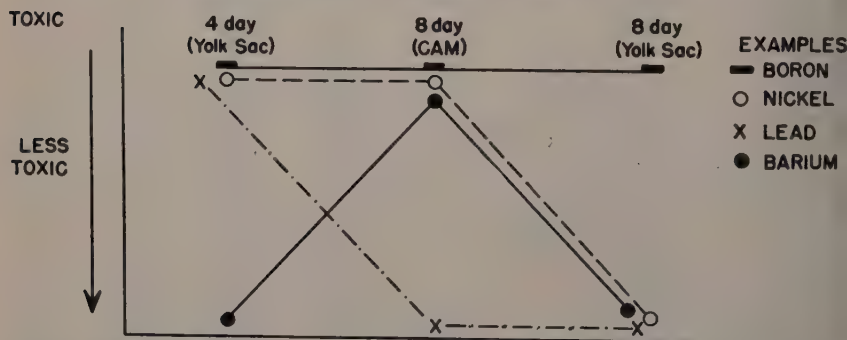


FIGURE 1. Toxicity of elements injected into four- and eight-day-old chick embryos by yolk sac and CAM routes. The four groupings cover the majority of results summarized in TABLE 1. The elements, illustrated by boron, require approximately the same LD₅₀ dose, irrespective of the age or route of administration. This may indicate that the chemical is readily absorbed, and that it acts specifically on a certain system as it appears in the embryo. If this system is not present at the time of injection the chemical remains biologically inactive until the proper susceptible mechanism appears. The elements, illustrated by nickel, show decreased toxicity at eight days as compared to four days by yolk sac injection, but they are toxic on the CAM at eight days. This may be interpreted as indicating that absorption of the salt is more efficient by the CAM route, and as the embryo increases in size more of the salt is necessary to poison it. About 16 per cent of the chemicals tested fell in this category. The elements, illustrated by lead, are most toxic at four days, and less toxic at eight days, by both yolk sac and CAM routes. This suggests that the salt is readily absorbed from the yolk sac as well as the CAM, and the tolerance of the embryo to the chemical increases with its increase in size. The elements, illustrated by barium, are more toxic by the CAM than by the yolk sac route. This pattern was found in about 50 per cent of the elements tested. Chemicals in this group may be precipitated in the yolk sac or they may react with the yolk components so that the embryo is not exposed to the full injected dose. Another possibility is that if the chemical is rapidly absorbed from the CAM, the embryo may be acutely susceptible to a high dose; although if the substance is absorbed slowly from the yolk sac, the embryo may detoxify or excrete it before a lethal concentration develops.

by any route of administration, while others are active only when given at specific stages of development, or by a certain route of administration, or in a definite chemical form. During the course of our toxicity studies, if gross and definite evidence of embryonic abnormality was produced by a given element, the effect was analyzed in more detail. If nothing specific occurred, possibly because an abnormality was missed by our gross methods of examination, or because the embryo was not treated properly to bring out such an effect, further studies have not been carried out. It does not mean, of course, that the element is completely without teratogenic activity. Nine elements thus far have been found to produce a specific and reproducible effect on embryonic development, and these are summarized in TABLE 2.

TABLE 2
SUMMARY OF ELEMENTS PRODUCING ABNORMALITIES IN EMBRYONIC DEVELOPMENT

Element	Abnormality	Maximum activity		Approximate consistency of effect, per cent treated embryo
		Ages of embryo at injection	Route of injection	
Thallium	achondroplasia	4-10	yolk sac, CAM	90-100
Chromium	mild achondroplasia	8	CAM	60
Lead	brain injury	3-15	yolk sac, CAM	80-90
Cobalt	brain injury	4-12	yolk sac	80-90
Boron	pale and edematous, feather inhibition	0-8	yolk sac, CAM	70-85
Arsenic	stunted, micromelia, abdominal edema	4	yolk sac	50
Rhodium	stunted, feather inhibition, micromelia, mild edema	8	CAM	80
Barium	abnormal feet	8	yolk sac, CAM	50
Selenium	bizarre abnormalities, stunting	0-8	yolk sac, CAM	10-20

(a) *Thallium*. Thallium sulfate produced a severe and consistent picture of achondroplasia in the chick embryo,¹ which was evident in embryos sacrificed or dying at ten days of incubation or later. The most marked effects were seen when thallium was given into the yolk sac at four to ten days of incubation, and the LD₅₀ dose during this period was between one and two mg/egg. Thallium did not produce achondroplasia when given at the maximum tolerated dose (0.3 mg/egg) to the two day embryo, presumably because thallium had a generally toxic effect at higher doses at two days, and lower doses were insufficient to produce achondroplasia. The LD₅₀ of thallium sulfate on the CAM at eight days is 0.7 mg/egg, and achondroplasia occurs consistently at doses of 0.4 to 0.7 mg/egg by this route. Thallium produces a less severe achondroplasia in embryos injected after the tenth day.

When sacrificed at 18 to 20 days, the embryo treated with thallium at four to eight days is severely achondroplastic, the limbs are short and the

long bones curved, the trunk is thickened, the head is small and the beak resembles a parrot's. On gross examination of the head, the eyes and orbit appear smaller than normal. The brain is small, sometimes contains cysts and the cerebellum may be atrophied. The abnormalities in the central nervous system are presumably due to compression of the brain and eyes as a result of defective growth of the skull. The feathers appear normal (FIGURE 2A). Roentgenograms of the thallium-treated embryos show the conspicuous interference in bone growth (FIGURE 2D, E). Microscopic section of the toe of the thallium-treated embryos shows defective cartilage growth, with failure of longitudinal bone growth, but the shaft is widened and thickened by the deposition of bone in the shaft (FIGURE 2B, C).

(b) *Chromium*. Sodium dichromate ($\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$) produced no apparent abnormalities when injected into the yolk sac of the four-day embryo. When sodium dichromate was injected onto the CAM, however, about 60 per cent of the embryos developed moderate achondroplasia, parrot beaks, slight stunting in growth, paleness and, in some instances, sparse feather formation (FIGURE 7C). Achondroplasia was never as severe as that produced by thallium. Injection of sodium dichromate into the yolk sac at eight days, and chromium nitrate ($\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$) into the four- and eight-day embryo failed to produce gross developmental abnormalities.

(c) *Lead*. The effect of lead on the early development of the chick embryo has been the subject of several studies, including those of Hammett and Wallace² and Catizone and Gray³; the latter group, working in the 0 to 48 hour of incubation period, concluded that lead can specifically affect the morphogenesis of the central nervous system of the chick. Our studies were carried out in older embryos, and lead was found to have a remarkable effect on the central nervous system. Four-day-old embryos injected with 0.15 mg. of lead nitrate showed an extreme degree of hydrocephalus when examined at 18 days of age⁴. In most instances, following the yolk sac injection of 0.15 mg/egg of lead nitrate at four days, embryos dying or sacrificed within the next four days exhibited brain hemorrhage (FIGURES 3A, B). Embryos examined after eight days of incubation presented varying degrees of hydrocephalus, ranging from a small herniation of the meninges to massive enlargement of the head (FIGURE 4A, B). After the tenth day of incubation, some of the brains showed evidence of regeneration of nervous tissue, particularly at the base and anterior portion.

Lead produced brain hemorrhage when injected into the yolk sac over the four- to 15-day incubation period. At eight days, doses of 0.1 mg/egg or higher of lead nitrate produced brain injury in 96 per cent of the embryos. These embryos usually showed necrosis of the brain substance at 18 days, and only a thin shell of nervous tissue remained, distended by a bloody fluid. Although the tolerance of the embryo for lead increased rapidly, the dose injuring the brain was fairly constant during the fourth to eighth day. At the tenth day and beyond, larger doses of lead nitrate, up to two mg/egg, were necessary to induce brain injury. This was attributed to the fact that other systems, such as bone, were appearing in the chick embryo, and the lead may have been competitively fixed by these new systems. The



FIGURE 2. Effect of thallium on the chick embryo. A—Embryo, sacrificed at 18 days, injected with one mg. of thallium sulfate into the yolk sac at four days. Embryo shows characteristic picture of achondroplasia, with shortened extremities and parrot beak. B—Microscopic section of toe of 18-day-old embryo. C—Toe of 18-day embryo treated at four days with thallium. The thallium-treated toe is shortened, cartilage formation impaired, but there is some bone formation in the shaft. D—Roentgenogram of a normal 18-day embryo. E—An 18-day thallium-treated embryo. The bones of the extremities are shortened, curved and appear denser than the control.

histopathology of the brain injury induced by lead is now under study,⁵ but the sequence of events, as they are seen grossly, are extensive hemorrhage throughout the brain substance within 48 hours after injection, followed by

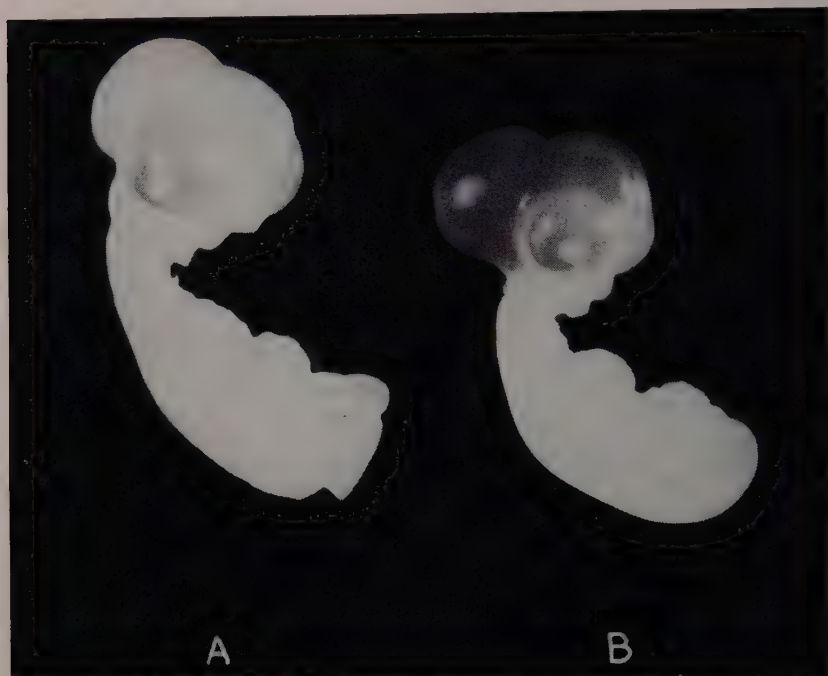


FIGURE 3. A—Control. B—Lead-treated embryo six days of age. Injected in the yolk sac with 0.15 mg. lead nitrate at four days of age. Marked hemorrhage is present in the brain.

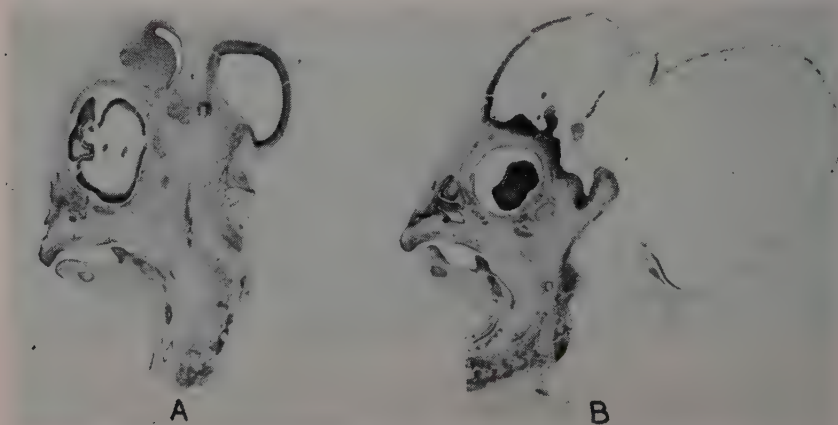


FIGURE 4. Microscopic sections of head of control A, and lead-treated embryo B, eight days of age. B was injected in the yolk sac with 0.15 mg. lead nitrate at four days. The cranium of the lead-treated embryo is enlarged and empty, as a result of the hemorrhagic necrosis in the brain induced by lead.

necrosis of the tissue. Some recovery is seen later, particularly if brain injury was induced early in development.

Lead nitrate failed to induce gross evidence of brain injury when injected into the yolk sac at two days of incubation, whereas in the three-day embryo, brain injury occurred in 70 per cent, and, in the four-day embryo, in 85 to 90 per cent of the treated embryos. It is of interest that the period of development when lead begins to induce its effects on the brain coincided with the appearance of blood vessels in the nervous system.⁶

The effects of lead and thallium could be induced by the simultaneous

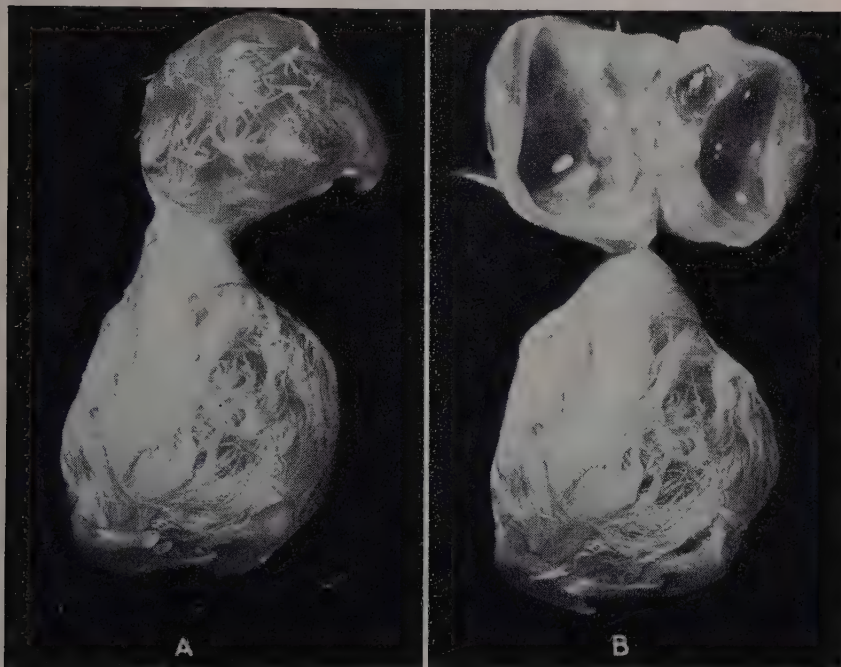


FIGURE 5. Embryo, 18 days of age, injected with one mg. of thallium sulfate into the yolk sac at four days, and 0.15 mg. of lead nitrate into the yolk sac at eight days of age. Note the achondroplasia and hydrocephalus (A), and the distended empty cranium (B). A similar picture followed the simultaneous injection of both agents at eight days of age.

injection of lead nitrate and thallium sulfate into the same embryo (FIGURES 5A, B).

(d) *Cobalt*. Gray and Scully⁷ reported that cobalt chloride, injected into the albumen of the egg before incubation, did not induce any specific abnormalities in the embryo, but it appeared to inhibit the growth of the amnion. In our work, cobalt has been used later in development. Cobalt chloride injected into the yolk sac of eight-day embryos consistently induced softening and some hemorrhage in the brain, when it was examined at 16 to 18 days (FIGURE 6A, B). The embryos also were pale, slightly edematous and the livers were enlarged. The nature of the brain injury is under study. Cobalt induced brain injury, apparently less severe than at eight days, when given at four or 12 days of development.

(e) *Boron*. When given in the yolk sac of the four-day-old embryo as boric acid, boron caused marked growth inhibition, and embryos surviving

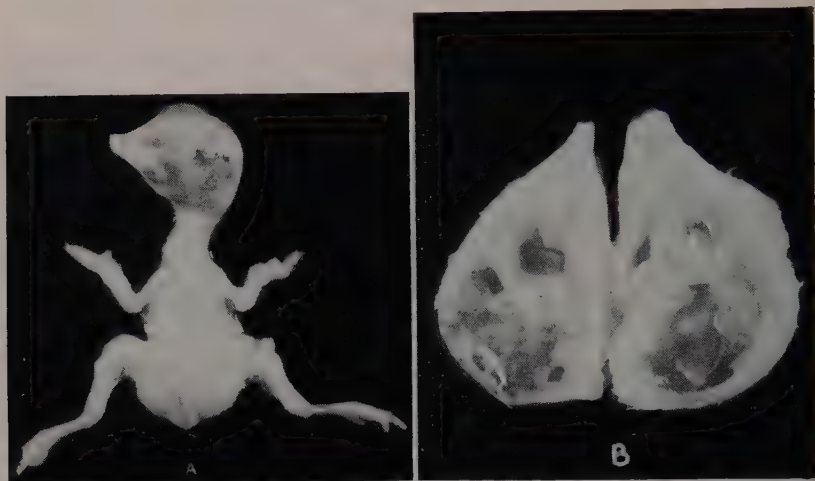


FIGURE 6. A—18-day-embryo, after injection in the yolk sac at eight days with two mg. cobalt chloride. Embryo shows redness and puffiness of the head, impaired feather growth, mild generalized edema and slight micromelia. B—Splitting the head reveals a soft brain with some hemorrhagic areas.

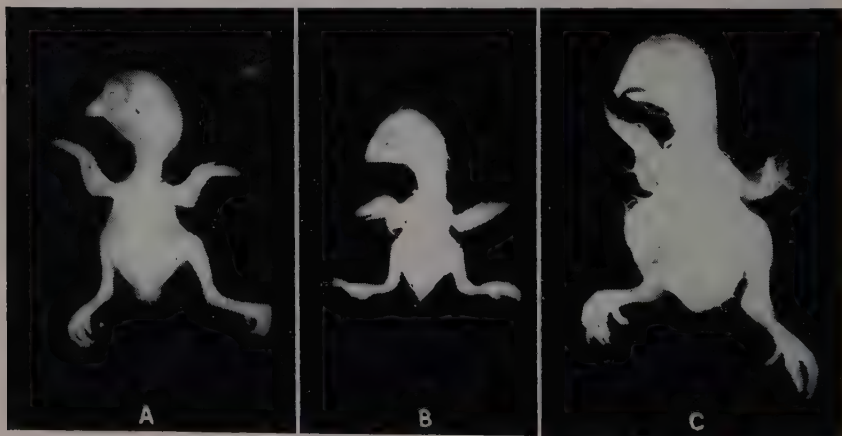


FIGURE 7. A—Embryo, sacrificed at 18 days, injected on the CAM at eight days with five mg. boric acid. The embryo is pale and stunted, and shows an over-all gelatinous edema and scanty and inhibited feather growth. B—Embryo, sacrificed at 18 days of age, was injected with two mg. of rhodium chloride onto the CAM at eight days. The embryo is severely stunted with mild micromelia and impaired feather formation. C—Embryo, sacrificed at 18 days, was injected onto the CAM with 0.1 mg. of sodium dichromate ($\text{Na}_2\text{Cr}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$) at eight days of age. The embryo shows a moderate achondroplasia with parrot beak, and slight growth inhibition.

to 18 days were also very pale, showed definite interference in feather growth and occasional beak deformities. Boric acid, by the yolk sac and CAM routes at eight days, caused a similar effect on the embryos, and those

surviving to 18 days also showed an over-all gelatinous edema (FIGURE 7A). The LD₅₀ dose of boric acid given into the yolk sac at zero and two days of age was in the same range as the four- and eight-day dosage, five mg/egg, and the effects on the embryo when examined at 18 days were somewhat similar, irrespective of the age of the embryo when injected. Boron, therefore, caused a specific and consistent effect on the chick embryo when given at zero to eight days at a fairly uniform dose level.

Arsenic. Sodium ortho arsenate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$), a pentavalent arsenical, produced definite growth inhibition when injected into the yolk sac



FIGURE 8. A—Embryo, sacrificed at 18 days, injected into the yolk sac at four days of age with 0.20 mg. of an arsenical salt ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$). The embryo is markedly stunted, shows mild micromelia, impaired feather growth and swelling of the abdomen, as compared with B, a normal 18-day-old chick embryo.

of four-day embryos, but not in the eight-day embryo. At 18 days, the embryos were small, showed mild micromelia and abdominal edema (FIGURE 8A). Potassium arsenite (KAsO_2) and arsenic pentoxide (As_2O_5) did not produce this picture when given at four or eight days.

Rhodium. Rhodium chloride produced abnormal embryos when injected on the CAM at eight days. The embryos surviving to 18 days were somewhat similar to those treated with sodium ortho arsenate, in that they were stunted, with mild micromelia and edema, but the rhodium treated embryos showed greater interference with feather growth (FIGURE 7B).

Barium. Barium chloride, injected into the yolk sac or onto the CAM of the eight-day embryo, produced a remarkable inhibition in the growth of the toes (FIGURE 9A). This abnormality occurred irregularly, but was seen in about 50 per cent of the treated embryos surviving to 18 days.



FIGURE 9. Feet of 18 day embryos. A—After injection in the yolk sac at eight days with 20 mg. barium chloride, and showing deformity and inhibition in toe development as compared with B—control normal toes.

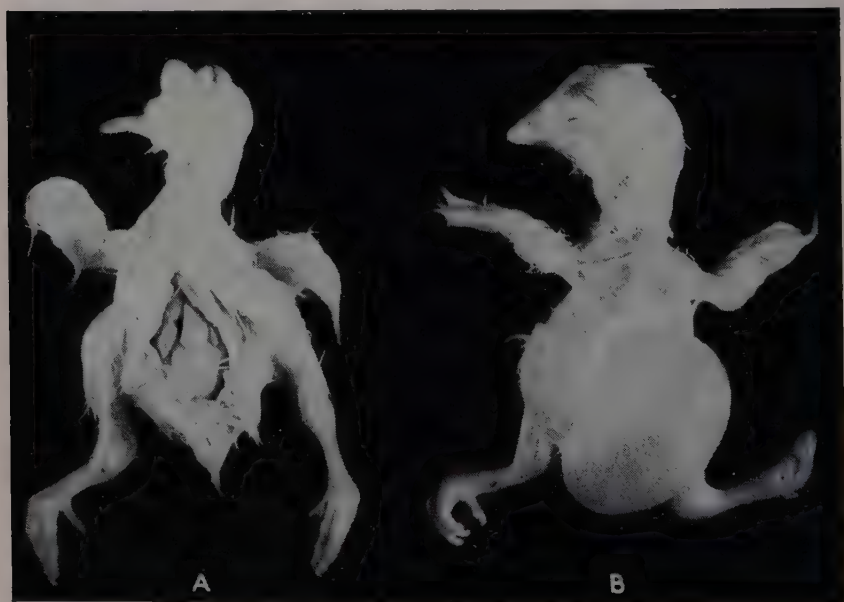


FIGURE 10. Effect of selenium on the chick embryo. A—18-day embryo injected in the yolk sac before incubation with 0.01 mg. selenious acid (H_2SeO_4). This embryo shows malformation of head and upper beak. B—18-day embryo injected in the yolk sac at eight days with 0.08 mg. of selenious acid. The embryo developed apparently normally, except for the enormous cysts on the rump.

There were no other gross effects noted on the embryo. None of the embryos injected at 4 days showed toe abnormalities.

Selenium. Franke, *et al.*⁸ obtained a variety of monsters following the injection of sodium selenate into the air sac prior to incubation. We obtained several abnormal embryos, two of which are shown in FIGURE 10A, B, when selenious acid (H_2SeO_4) was injected into the yolk sac of embryos from zero to eight days of age. The incidence of abnormalities in our experience was low, in the range of 10 to 20 per cent of the embryos surviving to 18 days.

Conclusion

The effects of excessive concentrations of elements on the developing chick embryo may be a useful method for studying the biological properties of the elements. It is apparent that some elements, at least, induce a characteristic effect on the embryo when given at the proper period of development, and two elements, given simultaneously, may each induce their specific abnormality in the embryo. Some of the elements have caused destruction and regression in an organ already well-developed at the time of treatment, thus demonstrating a teratogenic effect by retrogression of an established organ, rather than by damage to the anlage of a developing organ. The specific affinity of some elements and embryonic organs, as suggested by selective injury to these organs, may suggest approaches and methods for damaging neoplastic growths arising from those organs.

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THE EFFECTS OF RADIATION ON THE CHICK EMBRYO

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In considering the influence of Xrays and radioactive substances on embryonic development, it may be well to review briefly the manner in which ionizing radiations interact with tissues. The physicochemical basis of radiation injury is the ionization produced by the rays absorbed in or passing through the animal. This ionization causes changes in the cells which are primarily chemical. Ionization of the aqueous medium of the tissues forms oxidizing agents which interfere with many of the cellular enzyme systems, especially those utilizing free SH groups. The oxidizing effect is extremely short-lived, operating only during the period of radiation, but recovery of the affected enzyme systems may take considerable time. One of the most easily demonstrated effects of radiation is the inhibition of mitosis, especially important in the chick embryo, where all tissues show much mitotic activity. The interference with mitosis is presumed to be the result of interference with the nucleic acid cycle.

The chemical effects of radiation on cells and tissues are the same, regardless of the species or cell irradiated, and regardless of the variety of ionizing radiation. The biologic response to radiation may vary considerably, however, depending upon the particular characteristics of species or cell involved. With reference to the chick embryo, several of these biologic characteristics should be mentioned. First, all chick embryonic tissues have a high mitotic rate which is inhibited by radiation. Second, the sterile environment of the embryo eliminates infection, a complication of radiation injury which proves extremely important in some animals. Third, the ready supply of fluid and food in the egg may well minimize the dehydration and malnutrition which commonly follow post-radiation gastrointestinal damage in most animals.

One of the most practical criteria used in studying the biologic effects of radiation is the measure of the amount of radiation needed to kill. The lethal doses of radiation for chick embryos of various ages have been worked out in some detail by Dr. Karnofsky and his associates. The radiation doses which kill 50 per cent of embryos either within one day of radiation (acute) or within 10-15 days (delayed) are shown in TABLE 1. Acute deaths are dependent upon a relatively high dose rate of radiation, in this instance 43 roentgen per minute. The delayed lethal effects depend upon the total amount of radiation given, and are relatively independent of the rate of radiation. It is evident that very young embryos are resistant to the acute lethal effects of radiation. This resistance disappears, however, and by the eighth to tenth day of incubation the embryos are most sensitive to acute radiation effects with a continuation of this sensitivity throughout the rest of incubation. No definite explanation for this loss of resistance to the acute effects of radiation can be offered, but it would seem likely that, as the physiologic processes of the embryo become more complex, the embryo becomes more vulnerable to radiation. The simpler forms of life in general demonstrate greater radioresistance.

The amount of radiation necessary to produce a delayed lethal effect is relatively constant throughout incubation and even in the early weeks after hatching. These deaths presumably result from inadequate recovery and repair, for example the failure to replace blood cells, *etc.*

When radiation is given at extremely slow rates, the embryo is able to tolerate greater amounts. This is evident from the results of experiments we have carried out using intra-yolk injections with radioactive material, P^{32} , which give a continuous radiation of the embryo. As seen in TABLE 2, the total isotope roentgen equivalent doses tolerated by embryos which lived through incubation and some of which even hatched are several times the tolerated X-ray doses which are given over a much shorter time period.

TABLE 1
RADIATION MORTALITY— LD_{50} IN ROENTGENS

Age	Acute (1 day)	Delayed (10-15 day)
2-day embryo	>2000 (43 r/m)	1000-1200 (43 r/m)
5-day embryo	1500 (43 r/m)	1000-1200 (43 r/m)
8-day embryo	750 (43 r/m)	1200-1500 (3-6 r/m)
13-day embryo	800 (43 r/m)	1200-1500 (3-6 r/m)
18-day embryo	900 (43 r/m)	—
1-10-week chick	960 (43 r/m)	800 (43 r/m)
		1100-1200 (.28-10.5 r/m)

TABLE 2
RADIATION DOSES IN P^{32} -INJECTED EGGS

(RADIATION DOSES ARE ESTIMATED AND EXPRESSED AS ROENTGEN EQUIVALENTS)

P^{32} -injected	Day of incubation	Roentgen equivalents to hatching	% hatch
120 uc	4	4000	15
100 uc	8	2400	30
100 uc	14	900	50
Control	4-8	—	75

With this background of the general effects of radiation, we will consider the specific radiation-induced morphologic changes resulting from intra-yolk injection of P^{32} in chick embryos. The first facet of this study concerns the localization of P^{32} within the egg, whereby some idea of relative intensities of radiation in various tissues can be obtained. The relative concentrations of P^{32} in the components of the incubating egg are shown in TABLE 3. This simple experiment suggests the ideal combination of a closed system, such as the egg, and isotopes for use in tracer metabolic studies.

Further attempts to locate the P^{32} within the embryo reveal concentrations in the calcified bones 10 to 20 times those found in soft tissues. Because of this localization, and also because the beta emissions of P^{32} penetrate only a few millimeters in tissue, the bones and immediately adjacent tissues will receive the greatest amount of radiation.

The morphologic changes caused by the P^{32} radiation are, in the main,

similar to the effects of radiation in other species and are most obvious in the overall growth rate, gonads, bone, bone marrow, peripheral blood and lymphoid tissue.

The rates of growth of the radiated embryos decrease in proportion to the amount of radiation. Retardation of growth appears to be an overall effect with no one tissue or system more affected than another. There are no deformities or malformations attributable to the radiation, as have been reported by those using X-rays. It is likely that when large amounts of X-radiation are given over relatively short periods of time, those embryonic structures undergoing critical development coincident with the radiation may be malformed. Essenberg reported deformed or paralyzed legs in em-

TABLE 3
DISTRIBUTION P^{32} IN INCUBATED EGG
(APPROXIMATELY 5 MG KH_2PO_4 CONTAINING 20 uc P^{32} INJECTED INTO YOLK FIFTH DAY OF INCUBATION)

<i>Embryo</i>			<i>Allantois and amnion</i>	<i>Yolk</i>	<i>Albumen</i>	<i>Shell and membrane</i>
<i>Age of egg</i>	<i>Weight in gms.</i>	<i>% of total activity</i>	<i>% of total activity</i>	<i>% of total activity</i>	<i>% of total activity</i>	<i>% of total activity</i>
6	0.4	3	64	29	2	2
7	0.8	6	52	37	2	1
8	1.4	10	53	36	1	1
9	2.2	18	50	29	3	1
10	3.1	28	29	39	3	1
11						
12	5.8	58	21	19	1	1
13	8.1	65	12	19	3	1
14	9.3	71	12	17	.7	1
15	13.3	73	13	12	.8	1
16	16.9	73	16	7	.6	1
17	18.1	76	17	7	—	.8
18						
19	25.5	93	3	4	.3	.3
20	27.4	90	4	5	.4	.6

bryos X-radiated at 53-70 hours and suggested this time as a critical period in leg development. With continuous low rate radiation from P^{32} , such malformations are avoided.

The gonads are one of the most radiosensitive tissues of the chick embryo. In the testis the primary effect of radiation is destruction of the germ cells with no visible changes in the other elements. The destruction of germ cells is permanent, and after several weeks there is noticeable atrophy of the seminiferous tubules, independent of the amount of radiation. This tubular atrophy is probably secondary to loss of germ cells rather than a direct result of radiation. Male secondary sex characteristics fail to develop in all male chicks radiated during incubation. It appears that complete castration is effected.

The ova are the most radiosensitive elements of the ovary but their degree of radiosensitivity is dependent on the age of the embryo. In four- to eight-

day embryos, the ova can be completely destroyed by non-lethal doses of radiation. Complete destruction of ova by radiation is impossible, however, in embryos 14 days or more of age. The radioresistance of ova of young chicks is even greater than that of older embryos. The stroma of the ovaries shows no direct effects of radiation. There is, however, a delayed hypoplasia of ovarian stroma post-radiation which appears roughly proportional to the degree of injury to the ova and not to the amount of radiation. The normal atrophy of the right ovary post hatching shows simultaneous changes in all elements, while the radiation-induced ovarian atrophy shows first injury to the ova, followed later by stromal changes.

Radiation changes in the skeleton of the embryo are magnified because of the selective concentration of P^{32} in the calcified bones. There is a marked overall slowing of skeletal growth which appears to be proportional to the amount of radiation and is reversible after cessation of radiation. The rapidly dividing cartilage cells in the epiphyses and proliferating zones are the most radiosensitive. The first demonstrable changes are reduction in mitoses and irregularity in cell size and shape with a tendency to reduction in amount of intercellular hyalin. The proliferating zone becomes thin and is encroached upon by cartilagenous resorption from the shaft. Osteoblastic activity is reduced, as indicated by the thin cortical bone and almost complete absence of medullary bone spicules. Histologic recovery is prompt after radiation has stopped and growth of bone proceeds, without making up for the growth lost during radiation, however.

The bone marrow also receives heavy radiation from the P^{32} concentrated in the bones. In this experiment the bone marrow damage appears to be crucial so far as lethal effect is concerned. The first change caused by radiation is inhibition of maturation of the immature hematopoietic cells and the second change is a reduction of mitotic activity. With small amounts of radiation, these effects are transitory and recovery follows in several weeks. With radiation doses in or near the lethal range, these effects are prolonged and there is also destruction of hematopoietic cells and serous change in the marrow fat. The only cells of the marrow which do not show radiation change are the reticuloendothelial cells or hematopoietic stem cells. In severe radiation injury, the marrow may contain only a few stem cells, fat showing serous atrophy, and occasional pyknotic remnants of the hematopoietic cells. If the radiation disappears, recovery of the bone marrow proceeds from the remaining stem cells, which first appear to give rise to both early erythropoietic and myelopoietic forms. These small foci of regeneration show considerable mitotic activity and later the power of maturation returns, so that mature cells are finally formed.

The effect of this marrow injury is reflected in the reduction of all types of circulating blood cells. As can be postulated on the basis of radiation changes in the peripheral blood of young chicks, there is a prompt drop in lymphocyte count within a few days following radiation of the embryo, next a loss of granulocytes and finally, after a week or two, a decrease in red cells. These losses in the peripheral blood depend in degree and duration upon the amount of radiation; recovery occurs in the same order as injury, *i.e.*,

first a return of circulating lymphocytes, then granulocytes, and finally red blood cells. A contributing element in the development of radiation pancytopenia is the presence of numerous hemorrhages in most of the tissues of the embryo which accompany severe radiation injury.

Finally, lymphoid tissue is probably the most radiosensitive tissue of the embryo. Extremely small amounts of radiation stop the mitotic activity of these tissues. Moderate doses, well below the lethal range, produce cell destruction and fragmentation proportional to the amount of radiation. The recovery of these tissues post-radiation, as judged by observation of the thymus, is extremely rapid, and in some instances severe degrees of radiation injury are completely reversed and normal structure regained in two to three weeks.

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ENDOGENOUS VIRUSES IN THE EGG

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Introduction

The avian egg, during its formation and subsequent incubation, has been provided with many defenses that help to prevent invasion by viral and bacterial agents. Despite these protective devices, contamination of eggs can and does occur. Fortunately, however, the great majority of eggs are probably free from contamination. The widespread use of embryonated eggs for research and for vaccine production lends importance to those disease agents that are occasionally found within eggs.

There are two principal ways in which disease agents can gain entrance into the avian egg. One method is invasion during the process of egg formation, which is essentially a transfer of infection from the dam (and possibly the sire) to the egg. Thus, the various components of the egg, perhaps even the germ cells, may become infected. In addition, viable somatic cells from the dam that could harbor disease agents are frequently found within eggs, such as the cells in the blood spots.⁴⁵ The transfer of disease agents from the cloaca to the oviduct is another means of invasion of the egg. Even roundworms and flukes have been found within eggs.³²

The second way in which eggs can become infected is invasion after they have been laid. In this case the disease organisms may gain entrance into the egg through the shell. Fecal contamination of the shell from dirty nests is probably the main cause of this type of infection. The shell may be penetrated by disease agents at any time, however, even during incubation, if proper storage and incubator hygiene is not followed. It has been demonstrated that cultures of bacteria applied to the shell in a moist state can quickly penetrate the egg.⁵⁹ Recent work has shown that the practice of washing hatching eggs when properly carried out is not as dangerous from the standpoint of contamination as has been suspected.⁵⁴ The possible portals of entry for egg-borne diseases are illustrated in FIGURE 1.

Workers who employ embryonated eggs in their research may inadvertently encounter one of the egg-borne diseases of birds. The effect that these endogenous disease agents could have on the outcome of experiments has been suspected but never fully investigated. The endogenous bacterial diseases may be expected to produce deleterious results that would be obvious. Since the endogenous viral agents are more insidious, however, they may, for example, be carried along in serial passage with the known virus under study without the operator's knowledge, and subtle changes might result from this unwanted union. Similarly, vaccines prepared from embryonated eggs could become contaminated with these endogenous viral agents and escape detection.

The work of Delbrück²⁰ on the interference phenomenon in bacterial vi-

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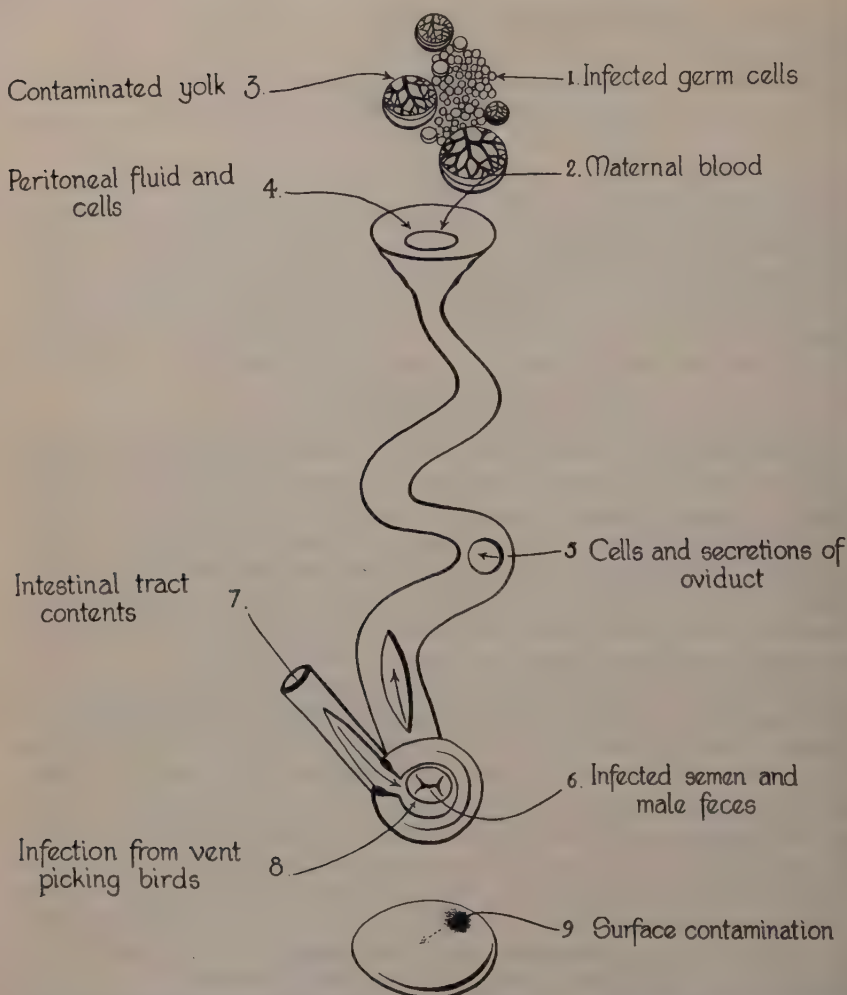


FIGURE 1. Schematic drawing of hen's reproductive system showing various ways disease agents may gain entrance into eggs.

Legend:

1. *Infected germ cells.* The female germ cells may carry disease agents into eggs.
2. *Maternal blood.* Some birds have a slight hemorrhage following ovulation. Thus, maternal blood, which could carry disease agents is often included in the egg, forming the so-called blood-spot.
3. *Contaminated yolk.* The yolk material accumulates over a period of several weeks before ovulation occurs. Disease agents could be deposited in the yolk and thus carried into the egg.
4. *Peritoneal fluid and cells.* Peritoneal infections can be carried into the egg via the infundibulum by means of peritoneal fluid and cells.
5. *Cells and secretions of oviduct.* The egg spends about 25 hours in the oviduct of the hen. During this time the chalazae, the albumen, shell membranes, shell and cuticle are added to the egg. An infection from the oviduct cells could be easily included in the egg.
6. *Infected semen and male feces.* The seminal fluid or spermatozoa could carry disease agents. In addition, feces from the male bird occasionally enters the female during copulation.
7. *Intestinal tract contents.* In many hens a physiological prolapse of the oviduct occurs at the time the egg is laid. This allows the everted wall of the oviduct to come in contact with the cloacal lining and contamination can thus be taken into the oviduct as it is retracted.
8. *Infection from vent picking birds.* Contamination on the bills of birds can be transferred to the oviduct of other birds that are the victims of cannibalism. The vent picking habit is quite common in laying hens, especially in groups which contain hens that are slow to retract their oviduct after laying.
9. *Surface contamination.* As explained in text, disease agents can readily penetrate the egg shell after the egg is laid.

rus clearly demonstrated some of the possible reactions when two different viruses were present in the same host. Findlay and McCallum²⁷ noted an interference phenomenon in experiments with Rift Valley fever and yellow fever. Following the work of Moore⁵¹ on the effect of Russian Far East encephalitis on transplantable mouse sarcoma 180, Sharpless *et al.*⁶¹ found that certain neurotropic viruses had an antagonistic action against transplants of lymphoid tumor strain RPL 12. Similar interferences between viruses, and reactions between viruses and tumors might be encountered when embryonated eggs containing endogenous viruses are inadvertently used for virus or tumor cultivation.

TABLE 1
DISEASES OF BIRDS TRANSMITTED THROUGH EGGS*

<i>Disease</i>	<i>Etiological agent</i>	<i>Principal hosts</i>
Avian lymphomatosis	Virus-like agent	Chickens
Avian encephalomyelitis	Virus	Chickens
Newcastle disease	Virus	Chickens
Infectious sinusitis	Virus	Turkeys
Psittacosis	Virus	Parakeets
Pullorum disease	<i>Salmonella pullorum</i>	Chickens
Avian tuberculosis	<i>Mycobacterium avium</i>	Chickens
Fowl typhoid	<i>Shigella gallinarum</i>	Chickens, turkeys, ducks
Paratyphoid infections	<i>Salmonella sp.</i>	Ducks, pigeons, chickens turkeys

* In most of the diseases listed above, the etiological agents have been found in eggs, but it has not been definitely established that all of these agents can cause disease in the hatched offspring as a result of egg transmission.

Egg-borne Diseases of Birds

This report is primarily concerned with the viral and bacterial diseases of birds that can be transmitted from dam to offspring through the egg.¹⁵ There are five viral diseases and four bacterial diseases of birds in which evidence suggests that egg transmission may occasionally take place (TABLE 1). The two most important diseases in this group from the egg transmission standpoint are avian lymphomatosis and pullorum disease. In addition to the poultry pathogens listed, many other types of bacteria have been isolated from eggs.⁵⁸

Avian Lymphomatosis. Avian lymphomatosis is an infectious, contagious, neoplastic disease of chickens. There are four forms of the disease, *i.e.*, visceral, neural, ocular, and osteopetrotic lymphomatosis. All of these forms, except osteopetrosis, are characterized by the invasion and multiplication of lymphoblasts (hemocytoblasts) in the affected tissues. It cannot be simply stated that all these forms of lymphomatosis are caused by a single virus, since the results of many transmission studies suggest that as many as three separate oncogenic virus-like agents may be involved. The evidence is more convincing that the strictly leukemic diseases, erythroblastosis and granuloblastosis, may add still another virus, separate from those of the various forms of lymphomatosis.⁶⁰ This unanswered question on the

unity or multiplicity of viral agents involved in lymphomatosis enters into any study that is made on the egg transmission of the disease.

The problem of egg transmission of lymphomatosis has been discussed for more than 20 years. Doyle,²¹ after making observations on the spread of neural lymphomatosis (fowl paralysis) in the Middle West during the late 1920's, was the first to suggest that the disease may be egg-borne. Soon afterward, other workers in England,^{48, 67} Australia,⁴⁹ Japan,²³ and elsewhere,⁴² made similar observations.

Since 1930 many workers have presented evidence both for and against the egg transmission hypothesis.¹² Solution of the problem has been difficult because of the contagious nature of the disease.⁷⁰ It has been shown that birds, from the day of hatching to about 30 or 40 days of age, are readily infected with the virus (or viruses) of lymphomatosis, either by natural exposure or by inoculation.^{9-11, 16, 69, 71} Furthermore, it has been demonstrated that natural exposure may take place via the respiratory route.¹⁶ The infected birds usually do not show clinical or gross evidence of the disease for many weeks following exposure, and ultimate expression of the disease, such as tumor formation and paralysis, may depend upon the stimulating influence of a "trigger" factor. A definite trigger factor has not as yet been discovered for lymphomatosis but there is suggestive evidence, however. In mammary tumors of mice such a trigger factor is operative, namely, the hormonal stimulation that leads to functional mammary glands.^{1, 7} This stimulus apparently sets the stage for mammary tumor development in the presence of the milk agent. Thus, the contagious and insidious nature of lymphomatosis makes it difficult to determine how chickens were infected with the disease—whether by direct or indirect contact or through the egg. The problem would be greatly simplified if disease-free stock was available. The disease is so widespread, however, that not a single poultry flock in the United States is known to be free from lymphomatosis. This disease has become the most important poultry disease problem in the nation, causing an estimated 75 million dollar annual loss.

The following different types of studies have given circumstantial evidence which suggested that lymphomatosis was transmitted through the egg:

(1) *Flock isolation studies.* Lymphomatosis developed in flocks that were started from hatching eggs introduced into new and isolated poultry quarters, such as the U. S. Regional Poultry Research Laboratory flock in East Lansing, Michigan, established in 1939,⁶⁹ the Regional Poultry Breeding Project in Lafayette, Indiana,⁶⁸ and the government sponsored flock in Southern Israel.⁴⁴

(2) *Family isolation studies.* Eggs from individual hens were placed in isolated incubators and the chicks were raised as family units in isolated pens. Some of the birds in these experiments developed lymphomatosis. Thus the disease apparently was brought in with the eggs.⁶⁹

(3) *Incubator exposure studies.* Groups of birds from a line showing a low incidence of lymphomatosis were exposed in the incubator at hatching time to other chicks from high incidence lines, and continuously exposed thereafter. These exposed chicks ultimately showed a significantly higher inci-

dence of lymphomatosis than did their sibs that were hatched in isolation and then exposed from that point onward, thus suggesting that incubator exposure contributes to the incidence of the disease and that egg transmission is implicated.⁷¹

(4) *Histological studies.* Ectopic lymphoid foci can be found in the tissues of certain embryos and newly-hatched chicks. Since Lucas *et al.*⁴⁵⁻⁴⁷ demonstrated that such lymphoid foci are statistically associated with lymphomatosis and may be the pre-tumorous lesions, the presence of these lymphoid foci in embryos may be additional presumptive evidence of egg transmission. Reyniers *et al.*⁵⁶ succeeded in raising in a germ-free environment a small number of Bantam chickens that were able to reproduce the second generation. Lymphoid tumors were not observed. The blood lymphocytes were reduced in number in the germ-free birds, and there was less lymphoid tissue along the intestinal walls and respiratory passages than in control birds. The number of lymphocytes in the thymus and spleen of the germ-free birds was not reduced.⁵⁶ It remains to be determined whether the birds in the germ-free environment are capable of developing avian lymphomatosis to the point where definite tumors are formed.

(5) *Transmission studies.* Studies that tended to indicate egg transmission were conducted by Durant and McDougle.²² They inoculated the blood from chicks that were the offspring of diseased parents (ocular lymphomatosis) into other susceptible chicks, and noted an increase in the incidence of neural lymphomatosis in the inoculated chicks.

Recently, Cottral *et al.*¹⁴⁻¹⁶ conducted studies on lymphomatosis in which embryo tissues and fluids were tested for the presence of a virus. They found direct evidence of egg transmission. Eight experiments were conducted, using inocula prepared from the tissues of newly-hatched chicks, tissues and fluids of embryos 15 and 18 days old, and blood and respiratory washings of certain selected donor hens. These tissues and fluids were tested for the presence of a virus by inoculating the material into groups of susceptible chicks (two to three days old) of an inbred line that had a low incidence of naturally occurring lymphomatosis. The sires and dams that furnished the eggs, and the chicks and embryos that supplied the inocula, were all clinically normal at the time they were used. Certain of the dams were used at random and others were selected because their sibs or raised offspring had shown either a high or a low incidence of lymphomatosis.

In the first two experiments pooled material from several families of chicks and embryos was used. Liver tissue from newly-hatched chicks, a cell-free filtrate of this tissue, and liver tissue from 15- and 18-day-old embryos were all found capable of transmitting lymphomatosis. The next step was to test individual hens by using liver tissue from their embryonic offspring. Five of twelve hens tested, which had been living in an environment with maximum exposure to naturally-occurring lymphomatosis, were found to be producing infected embryos. Retests on four of these twelve hens gave the following results: hen 709W, positive on both tests; hen 310R, negative on both tests; hen 716R, negative on first test, but positive on second for both embryo liver tissue and amnionic fluid; and hen 430H, positive

on first test and negative on second. When five of the twelve hens were tested, using heparinized whole blood from the hens as the inoculum, only two gave positive results, and one of these had been negative by the previous embryo test. Respiratory washings from two of the hens were tested, but the results did not indicate transmission. Tests also were made of other hens that had been hatched and reared in an isolated environment in which a low incidence of lymphomatosis had been observed. Two of these nine isolated hens were found to be producing infected embryos. The results on one of these hens was on the border line of significance, however. Retests were made on five of the non-transmitting isolated hens and the results agreed with those of the previous test.

An interpretation of these experiments was difficult because of the presence of naturally occurring lymphomatosis in the various non-inoculated contact control groups, in which the incidence varied from 5.3 to 26.1 per cent. The percentage of lymphomatosis in the treated groups varied from five to 100 per cent, and the only groups that were considered as indicating transmission were those that showed a significant chi-square value at the five per cent level or less. Thus, between the significant treated groups and their respective controls, there was a difference in incidence of 30 per cent or more. Visceral and neural lymphomatosis were observed in both the treated and the control groups. Only visceral lymphomatosis was significantly increased in the inoculated birds, however. In addition, six cases of osteopetrosis occurred in the inoculated birds, and none in the control groups.

The results of these experiments, in conjunction with the evidence that has been gathered in the past, lead to the conclusion that lymphomatosis, especially visceral lymphomatosis, is transmitted through the egg from normal-appearing parents to their offspring, a form of disease transmission that Gross has labeled "vertical transmission."²⁹ Thus, clinically normal hens, with no gross evidence of tumors, can be carriers of a virus which will produce a neoplastic disease: visceral lymphomatosis. These findings closely parallel the observations made on mammary tumors in mice, in which the milk factor may be carried and transmitted to the suckling offspring by clinically normal female mice.^{1, 7}

It cannot be determined from these experiments how many hens in the general population may be carriers of lymphomatosis. Recently, Cole and Hutt¹³ presented evidence that egg transmission was not an important factor in the spread of lymphomatosis in their flock. This problem cannot be settled easily because there appear to be great differences in the incidence and type of lymphomatosis in the various poultry flocks throughout the United States. Furthermore, the fact that a group of birds raised for part of their lives in isolation did not show gross tumors of lymphomatosis until they were removed from isolation, does not prove that the virus was not transmitted to them while in isolation. An analogy may be drawn to mammary tumors in mice. If mice infected with the milk agent are prevented from developing functional mammary glands, very few, if any, mammary tumors will result.^{1, 7} Thus, we cannot appreciate the full import of egg transmission in lymphomatosis until we learn more about the factors, other than the virus, that help to bring about the tumor formation.

In the ideal situation for the perpetuation of a host-pathogen relationship, so that transmission from one generation to the next can occur easily, the pathogen should not harm the embryonic host. The virus of lymphomatosis seems to fill this requirement, because no detectable lesions are produced when embryos are inoculated with the virus. Suspensions of lymphoid tumor cells will grow and produce tumors in embryonated eggs, but cell-free extracts are without visible effect on the embryo.

Avian Encephalomyelitis. Jones³⁹ was the first to report (in 1932) the presence of this virus disease of chickens. His early observations led him to believe that the disease was not egg-borne. Later Van Roekel *et al.*⁶⁵ concluded that the disease was egg-borne. Jungherr and Minard⁴⁰ concurred with that observation and presented evidence that the virus persists in the visceral tissues of certain adult birds, and may be eliminated via the genital or intestinal tract. Recently, Feibel²⁴ found that avian encephalomyelitis virus survived for five to six weeks in the yolk sac of chicks inoculated via that route as embryos. The virus also survived in the allantoic fluid of embryonated eggs for six to seven days post-inoculation.

The workers at Notre Dame⁵⁷ encountered, in germ-free chicks, a disease that occurred 12 to 48 hours after hatching and which they called "Jitters." The disease was found in two groups of chickens hatched in the germ-free environment, but was not observed in the contaminated control chicks from the same flock. The disease could not be produced in contaminated control chicks by inoculation. In the germ-free environment, the disease was arrested when the birds became contaminated with a pure culture of bacteria. Thus, the suspected virus seemed to require a bacteria-free host for the exhibition of symptoms. The symptoms described are similar to those of avian encephalomyelitis, but "Jitters" may be an entirely unrelated disease and egg-borne.

The concept of egg transmission of avian encephalomyelitis rests entirely on circumstantial evidence. This concept is somewhat strengthened by the fact that the disease usually affects chicks shortly after hatching (birds from one to three weeks old). The concept is also strengthened by the inability of various workers to prove conclusively that contact transmission takes place under field conditions.⁵² The disease has a very sporadic occurrence and apparently is seasonal (winter and spring).⁵² Little can be done to control it.

Newcastle Disease. Newcastle disease, or pneumoencephalitis, has been studied intensely during the past eight years. Brandy *et al.*⁸ demonstrated the presence of antiviral antibodies in the yolk of eggs laid by immune, recovered hens. At approximately the same time, other workers were able to isolate the virus of Newcastle disease from fresh eggs laid by infected hens and from the ovarian tissue of such hens.^{3, 41, 66} DeLay¹⁹ reported isolation of the virus from the yolk sac of four-day-old chicks, dead embryos, and infertile eggs from infected parent stock. Hofstad³⁵ also found that dead embryos and infertile eggs contained the virus when the eggs from infected hens were examined during the decline in egg production following an outbreak of the disease. The virus could not be detected in the first eggs laid by such hens when they resumed production, however. Beaudette⁶

was unable to detect the virus in dead embryos and infertile eggs laid by hens that had been vaccinated with a live virus vaccine.

The evidence indicates that Newcastle disease virus can be transmitted to the egg, especially when the hens are in the period of declining production following an outbreak of the disease. Due to the embryonic mortality caused by the virus, however, apparently only a few infected embryos would survive to hatching time. The use of live virus vaccine to protect laying flocks apparently does not result in egg transmission of the virus.⁶

Infectious Sinusitis. Several groups of workers recently have demonstrated that infectious sinusitis of turkeys is caused by a virus.^{30, 34, 36} Furthermore, Jerstad and Hamilton³⁶ found evidence of egg transmission of the virus. The virus was isolated from turkey embryos that were the offspring of infected breeders. These workers later were able to demonstrate the virus in poults that had pipped and died and in two poults that died eight and 14 days after hatching.³⁷ Experiments in which chick embryos were inoculated with infectious material showed that some would survive and develop the disease several days after hatching.³⁴

Grumbles and Boney³¹ stated that field observations suggested egg or hatchery transmission of infectious sinusitis, but they were unable to demonstrate the virus in eggs from the infected breeders they examined.

The virus has been cultivated in chick embryos, and experiments have shown that it will survive to hatching time and for several days post-hatching.³⁴ The work of Delaplane¹⁸ suggested that the virus of infectious sinusitis of turkeys was identical with the virus causing chronic respiratory disease of chickens. The inoculation into turkeys of the virus isolated from chickens produced typical infectious sinusitis symptoms in the turkeys. No evidence of egg transmission of chronic respiratory disease of chickens has been reported. The evidence seems to indicate that egg transmission of infectious sinusitis virus can occur, but it is not easily demonstrated because of technical difficulties encountered in isolation of the virus, and because of the long incubation period for the disease.³⁷ It seems likely that egg transmission of this viral agent does not often occur. Some workers have had good field results in controlling sinusitis in poults, however, by eliminating infected breeder flocks.³⁸

Psittacosis and Ornithosis. Meyer⁵⁰ presented evidence that psittacosis virus could be egg-borne in parakeets. He found that the virus could be detected in the ovaries and eggs of infected parakeets, and observed that young birds, even nestlings, showed active infection with the virus. More recently, Karrer *et al.*⁴³ have found that the virus can be detected in pigeons and chickens. The ornithotic infection in these birds is even less obvious clinically and pathologically than is the comparable disease in the psittacine birds. It is not known to what extent chickens are infected with ornithosis, or whether the virus is transmitted through the egg in chickens. Davis and Vogel¹⁷ inoculated embryonated chicken eggs with psittacosis virus, however, and demonstrated that the agent survived and could be detected as long as 22 days after hatching. With many wild birds as carriers of the virus, the disease may ultimately become a poultry problem. The insidious and latent characteristics of the disease make it a difficult one to detect and eliminate.

Pullorum Disease. In 1909, Rettger and Stoneburn⁵⁵ isolated *Salmonella pullorum* from fresh and incubated eggs that were laid by hens whose other progeny had previously developed pullorum disease or bacillary white diarrhea. This was the first demonstration of an egg-borne disease of poultry. Many other investigators have confirmed these findings and have demonstrated that *S. pullorum* may be present in many eggs laid by carrier hens. Furthermore, the bacteria can be isolated from the ovaries of such hens.⁵⁵ Pullorum disease has been a major problem to the poultry industry, but the disease is gradually being brought under control by the widespread use of the agglutination test, which points out the carrier hens to be eliminated from the flock. Investigators using embryonated eggs in their research can circumvent trouble from this disease by obtaining hatching eggs only from approved pullorum-free flocks.

Avian Tuberculosis. In 1890, Sibley⁶³ found avian tuberculosis in chickens that had been hatched from eggs laid by hens affected with the disease. Fitch *et al.*²⁸ made bacteriological studies of the eggs laid by tuberculous chickens and found that less than one per cent of the eggs were infected. Thus, the organisms can enter the eggs, but, from the disease dissemination standpoint, as pointed out by Feldman²⁵ in his recent review of the subject, egg transmission of tuberculosis apparently does not constitute a serious problem to the poultry industry, since many hundreds of chickens have been hatched from eggs of naturally infected hens without tuberculosis being observed. The active campaign waged against avian tuberculosis has succeeded in greatly reducing the incidence of the disease. Numerous flocks thus are free of the disease.

Fowl Typhoid. In 1925, Beaudette⁴ suggested that fowl typhoid may occasionally be transmitted through the egg. He succeeded in isolating *Shigella gallinarum* from the unabsorbed yolk sac of newly-hatched chicks and from the ovaries of adult hens in the flock furnishing the infected chicks. Similar observations were made by Beach and Davis.² Later, Beaudette⁵ isolated the bacteria from the yolk sac of a dead embryo. He also pointed out that the epidemiologic evidence in many instances indicated egg transmission. The recent work of Hall *et al.*³³ demonstrated that egg transmission occasionally occurred in this disease, but the other methods of transmission appeared to be more important in its dissemination. Anyone using embryonated eggs would have no great difficulty in locating supply flocks that are free of fowl typhoid.

Paratyphoid Infections. The paratyphoid infections in birds are caused by the *Salmonella* group of organisms. There are many serologically related bacteria in this group and some of these organisms can cause food poisoning in man.²⁶ In addition to chickens, many of the other avian species are also affected. Fenstermacher²⁶ recently has reviewed the literature concerning the isolation of these organisms from various species of birds. The work of Schalm⁵⁹ and many others²⁶ indicated that the paratyphoid organisms could penetrate the eggshell and that infection of the egg probably took place after it had been laid. Whether eggs carry paratyphoid organisms externally or internally, they have presented a public health problem in some localities, and duck eggs have been the chief offenders.

Discussion and Summary

The knowledge that endogenous disease agents occasionally are present in eggs is not a new finding, nor is this form of disease transmission peculiar to birds. Egg transmission of disease agents occurs in certain arthropod and helminth vectors, as shown in TABLE 2. There are also seed-borne diseases of plants.¹⁵

Pasteur⁵³ was the first to demonstrate egg transmission in his classical work on pébrine, a protozoan disease of silkworms. Later, Smith and Kil-

TABLE 2
DISEASES PERPETUATED IN ARTHROPOD AND HELMINTH VECTORS BY EGG TRANSMISSION*

Disease	Etiological agent	Vector	Host affected
Texas fever	<i>Babesia bigemina</i>	<i>Margaropus annulatus</i> (tick)	Cattle
Rocky Mountain spotted fever	<i>D. rickettsi</i>	<i>Dermacentor andersoni</i> (tick)	Man
Boutonneuse fever	<i>D. conori</i>	<i>Rhipicephalus sanguineus</i> (tick)	Man
Scrub typhus	<i>R. tsutsugamushi</i>	<i>Trombicula akamushi</i> (mite)	Man
Phlebotomus fever	Virus	<i>Phlebotomus papatasi</i> (fly)	Man
Colorado tick fever	Virus	<i>Dermacentor andersoni</i> (tick)	Man
St. Louis encephalitis	Virus	<i>Dermanyssus gallinae</i> (mite)	Chickens
Equine encephalomyelitis, Western	Virus	<i>Dermacentor andersoni</i> (tick)	Horses
Dwarf disease of rice	Virus	<i>Nephotettix apicalis</i> (leaf hopper)	Rice
Fowl spirochaetosis	<i>Treponema anserinum</i>	<i>Argas persicus</i> (tick)	Chickens
Enterohepatitis	<i>Histomonas meleagridis</i>	<i>Heterakis vesicularis</i> (cecal worm)	Turkeys
Swine influenza	Virus	<i>Metastrogylus sp.</i> (lung-worm)	Hogs
Lymphocytic choriomeningitis	Virus	<i>Trichinella spiralis</i> (Trichina)	Guinea pigs

* For references to the original work on these diseases, see Cottral.¹⁵

bourne⁶⁴ studied Texas fever of cattle and demonstrated that the organism, *Babesia bigemina*, is transmitted from bovine to bovine only through the agency of the offspring of female ticks which have previously fed upon the blood of infected cattle. Since then, many other disease agents have been proven to be perpetuated by egg transmission in certain helminth and arthropod vectors.¹⁵

The perpetuation of a disease agent by direct transmission from one generation to the next has been called aptly "vertical transmission." As Gross²⁹ has pointed out, mouse mammary carcinoma and, also, mouse leukemia are examples of "vertical transmission" in mammals. Recently, Shay *et al.*⁶² reported on the development of malignant lymphoma in young rats suckled

by mothers receiving methylcholanthrene by stomach tube during the lactation period. This work indicated that carcinogens might also be transmitted from one generation to the next.

It has been shown that nearly all types of disease producing agents, including protozoa, fungi, bacteria, rickettsia, viruses and carcinogens, may be transmitted from one host generation to the next through eggs, seeds or milk. It was pointed out that the endogenous viruses and bacteria found in avian eggs can gain entrance either during the process of egg formation or after the egg has been laid. Thus, the term "endogenous" has been used in its broadest interpretation. The five viral and four bacterial diseases of birds in which the evidence indicates that egg transmission may occasionally take place were discussed and listed as follows: avian lymphomatosis, avian encephalomyelitis, Newcastle disease, infectious sinusitis, psittacosis, pullorum disease, avian tuberculosis, fowl typhoid, and paratyphoid infections. In some of these diseases, it has been shown that egg transmission is the primary mode of spreading these diseases, while others are only rarely egg-borne and may produce disease in the hatched offspring only occasionally. The evidence indicating the occasional presence of these disease agents in avian eggs was quite conclusive for certain of the diseases, but it was mainly circumstantial for others.

Workers who employ embryonated eggs in their research or use them for vaccine production should be aware of the possibility that these endogenous disease agents could be present in the eggs that they use, and that these unwanted agents might interfere with, or influence the experimental results. In live virus vaccine production, the endogenous viral agent might be carried along as an unknown contaminant that could conceivably produce a disease in the recipients of the vaccine. Some of the virus interference phenomena and viral antagonisms in tumors were mentioned as examples of what might happen in experiments in which endogenous viruses were present in embryonated eggs used for virus or tumor propagation.

Workers should obtain their experimental eggs only from well-managed hatcheries and supply flocks, preferably from those establishments that maintain a closed flock and are constantly on guard against the various poultry diseases. Good storage and incubator hygiene should be practiced to avoid the possibility of accidentally contaminating the eggs in the laboratory, because disease agents can readily penetrate the egg shell.

Of the various egg-borne diseases of birds, avian lymphomatosis appears to be the most difficult to eliminate. Unfortunately, the extent to which this virus may be present in eggs is not known, and the harm, if any, that it can do to experiments employing embryonated eggs remains undetermined. A method for controlling this disease has not been found. Control measures are being used for some of the other egg-borne diseases. The egg-borne bacterial diseases are easily controlled in supply flocks. Studies at the University of Notre Dame indicated that approximately 95 per cent of the eggs used were free of viable bacteria.⁵⁶ The evidence is inadequate to make a comparable statement regarding the egg-borne viruses. Thus, the occasional presence of these endogenous disease agents in avian eggs challenges the customary concept that eggs are sealed, germ-free systems.

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Discussion

DR. EDGAR ZWILLING (*University of Connecticut, Storrs, Conn.*): If egg infection is so widespread, this must interfere with virologists' carrying pure strains of viruses in eggs. Do you know how often, or in how many cases, pure strains are lost because of egg-born contamination?

DR. COTTRAL: That sort of information is difficult to get. People do lose pure strains but frequently don't know the source of infection. I'd say that we really don't know much about this.

DR. HERALD R. COX (*Lederle Laboratories, Pearl River, N. Y.*): Dr. Cottral has just pointed out the possibility of such viruses as Newcastle disease, infectious bronchitis and laryngotracheitis being present as latent or unrecognized infections in certain chick embryos, and how it might be possible to disseminate such viruses as contaminants in living virus vaccines used in either veterinary or human medicine. I wish to state that in actual experience, wherein literally millions of eggs have been used for the production of such vaccines, never have we encountered a virus agent as a contaminant that could be detected by the various types of safety tests that we must necessarily employ for release of such products. Thus, on the basis of actual tests, we believe the chances are rather remote of picking up such viruses as contaminants in the chick embryo tissues being used for vaccine production. Furthermore, it would appear that nature has pretty well provided that such an event should not occur, since all of the viruses mentioned would, in all probability, cause death of the developing chick embryo before it ever

reached an age where it could be used satisfactorily in vaccine production. It must be kept in mind that in the production of living viral vaccines, the embryos must be living not only at the time of inoculation but also at the time of harvest, so that by following these simple precautionary rules, the possibility of picking up a virus agent known to be pathogenic for chicks seems rather remote. I know that the experiences of Dr. Buddingh in his laboratory fully confirm my own. As a matter of fact, I know of no instance as yet wherein the chick embryo has been shown to be a natural carrier of any virus agent.

GROWTH OF VIRUSES AND RICKETTSIAE IN THE DEVELOPING CHICK EMBRYO

By Herald R. Cox

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Those working in the field of viruses and rickettsiae know that the developing chick embryo is one of the most valuable and widely used media for the propagation of this group of infectious agents. It is equally valuable for investigational work and for the commercial production of prophylactic and diagnostic agents. As a matter of fact, only the white mouse can be considered comparable to the chick embryo in its general use and adaptability. It would be difficult indeed to over-emphasize the usefulness of either of these hosts in facilitating quantitative studies and in other ways furthering our knowledge of this most interesting and important group of infectious diseases.

It appears that the first use of the developing chick embryo for the experimental study of a viral agent was the work of Rous and Murphy in 1911,¹ who inoculated tissue suspensions and cell filtrates of chicken sarcoma No.1 (Rous sarcoma) into developing hen's eggs at seven to eight days incubation. Tumors were found to develop in the various tissues injured by injection, with the most frequent occurrence of tumors being on the chorio-allantoic membrane. The same procedure was used by Murphy² for propagating mammalian cells of the Jensen rat sarcoma on the chorio-allantoic membrane. Other contributions along the same lines were those of Jouan and Staub in 1920 with fowl pest³ and Gay and Thompson in 1929 with vaccinia virus.⁴

Major credit today, however, is given to Goodpasture and his associates for first emphasizing in 1931⁵ the tremendous possibilities in the use of developing chick embryos for "research work and in practical applications to prevention and treatment." Using the method described by Clark⁶ for embryological work, Woodruff and Goodpasture⁵ used the virus of fowl-pox in their first study, in which they obtained well-marked lesions characteristic of the disease on the chorio-allantoic membrane. Shortly thereafter, Goodpasture, Woodruff and Buddingh^{7, 8} reported on infection of the chorio-allantoic membrane with the viruses of vaccinia and herpes simplex. In 1933, Burnet⁹ described a modification of Goodpasture's method of inoculation, in which an artificial air sac was produced above the chorio-allantoic membrane so that a relatively large volume of inoculum could be used. This procedure greatly facilitated the pock-counting titration studies on the chorio-allantoic membrane which Burnet and his colleagues used so extensively in studying such viruses as vaccinia,¹⁰ herpes simplex¹¹ and ectromelia.¹² Again in 1934 Burnet and Ferry¹³ reported that the virus of fowl plague rapidly killed chick embryos. In addition, the viruses of vesicular stomatitis and Newcastle disease were found to be fatal. These findings indicated a new and more definitive method in which the chick embryo could be used for virus titration studies, *i.e.*, the death of the embryo as proof of infection. Space does not permit a review of the extensive work of the

great number of investigators who have subsequently employed the developing chick embryo in studying practically all the viral and rickettsial diseases recognized today. Those interested in the history of the development of the chick embryo method as well as detailed accounts of the various techniques involved should consult the publications of Goodpasture and Buddingh,¹⁴ Burnet,¹⁵ Goodpasture,¹⁶ Polk, Buddingh and Goodpasture,¹⁷ and Beveridge and Burnet.¹⁸

Methods of Inoculation

TABLE 1 shows the various methods of inoculation that have been devised, together with the names of the original investigators and the dates of publication.

Applications of the Various Techniques

Chorio-allantoic Membrane. The chorio-allantoic (C-A) membrane technique, which was first reported by Woodruff and Goodpasture in 1931 for

TABLE 1
METHODS OF INOCULATION

TABLE 2
FACTORS INFLUENCING THE GROWTH OF
VIRUSES AND RICKETTSIAE

Chorio-allantoic membrane (Woodruff and Goodpasture, 1931)	Age of embryo
Amniotic sac (Gallavan and Goodpasture, 1937)	Route of inoculation
Embryo stab (Elmendorf and Smith, 1937)	Dilution and volume of inoculum
Yolk sac (Cox, 1938)	Temperature of incubation
Intravenous (Polk, Buddingh and Goodpasture, 1938)	Time of incubation following inoculation
Intracerebral (Dawson, 1939)	Vitamin deficiency
Allantoic sac (Burnet, 1941)	Use of enzyme inhibitors or activators
Intra-ocular (Dawson, 1941)	

the study of fowl-pox virus, has been found by many subsequent workers to furnish a very satisfactory method by which to study the development of inclusion bodies in various virus infections. Such inclusion bodies characteristic of the following diseases have been found in stained preparations of the chorio-allantoic membrane: psittacosis, vaccinia, lymphogranuloma-venereum, herpes simplex, equine encephalomyelitis, fowl-pox, ectromelia, Kikuth's canary virus, infectious laryngotracheitis of fowls and Pacheco's parrot disease.

Grafts of tissues already infected with virus, of human or animal origin, may be placed on the C-A membrane and the development of inclusions may then be studied by regular histological procedures.¹⁹

A large number of human and animal viruses can be cultivated readily by serial passage on the C-A membrane. Generally, subculturing may be carried out by grinding the infected membrane, centrifuging it lightly, and injecting the supernatant fluid on to the C-A membrane of fresh eggs. Animal or other titrations may be carried out to prove that there is an actual increase in the virus content of the membrane.

The C-A membrane technique may be employed for titration of viruses, using either death of the embryo or the pock counting technique to determine the end-point, depending upon the virulence and other characteristics of the particular virus being studied.

Certain viruses, such as influenza, mumps and variola, may be isolated by direct inoculation of the C-A membrane.

Antiviral sera for such agents as vesicular stomatitis, herpes simplex, vaccinia, variola and infectious laryngotracheitis of fowls may be titrated for antibody content using the C-A membrane technique. Again death of the embryo or suppression of pock formation on the membrane determines the end-point.

C-A membrane route of inoculation is the method of choice for production of such vaccines as vaccinia, fowl-pox and pigeon pox. In addition, this technique has been used as a means of studying the theory of antigen-antibody reactions²⁰, as a source of antigen in the preparation of diagnostic antigens, and as a tool for the testing of chemo-therapeutic agents.

Amniotic Sac. The chief value of the amniotic sac route of inoculation is to introduce material so that it will come in contact with tissues of the respiratory tract. This method has been used particularly in work with influenza virus. Burnet has employed the amniotic sac route to retain the human 0 phase characteristics of the BEL strain of influenza virus by passing the strain at its limiting infective dilution.²¹ In all probability, Burnet very rightly regards this combined procedure to be of "crucial importance" to the genetic approach of influenza viruses and he has used it as mentioned above to maintain "pure clone" strains of the 0 phase type.

Allantoic Sac. The entodermal lining of the allantoic cavity is quite susceptible to a number of viruses, such as influenza, mumps, Newcastle disease, laryngotracheitis virus of fowls and the equine encephalitis viruses, and the method of allantoic sac inoculation is widely used. The principal applications of this route of inoculation are: (1) passage of laboratory strains; (2) primary isolation from human and animal source materials; (3) source of virus that can be partially purified for morphological and chemical tests; (4) source of antigen in preparation of vaccines and for serological tests; (5) method for titrating virus suspensions or determining the antibody content of antisera; and (6) substrate in chemotherapeutic experiments.

Yolk Sac. The yolk sac route of inoculation has been found to be one of the most satisfactory methods to employ in chick embryo work, partly because of its great simplicity in technique and partly because the yolk sac cells are amongst the most susceptible of any in the embryo to virus, rickettsial, bacterial and mycotic infections. Furthermore, the yolk sac route is one of the choice methods, if not the best, to establish virus infection of the embryo. This method of inoculation was first shown by Cox²² to be particularly useful for growing rickettsiae of all types, such as those of the Rocky Mountain spotted fever, typhus fever, Q fever and scrub typhus groups. The method has been used extensively on a practical basis for production of such vaccines as Rocky Mountain spotted fever, epidemic and murine typhus fever, Q fever, rabies, canine distemper, Japanese B encephalitis and the

equine encephalitis viruses. In addition, it has been used for producing diagnostic antigens for rickettsiae and viruses of the psittacosis-lymphogranuloma group; for titrating virus suspensions or determining the antibody content of antisera; and in chemotherapeutic assays.²³ Recently, the yolk sac route has been shown to be satisfactory for the propagation in serial passage of such exotic viruses of African and South American origin as Ntaya, Bwamba, Bunyamwera, Uganda S, Zika, Haemagogus A, Haemagogus B, Leucocelaenus, Sabethes,²⁴ Semliki,^{24, 25} Mengo,^{24, 26} West Nile,^{24, 27} Anopheles A,^{24, 28} and Ilheus.^{24, 29}

Intracerebral Inoculation. The cells of the embryonic chick brain are susceptible upon intracerebral inoculation to infection with the viruses of herpes simplex³⁰ and rabies.³¹ It should be mentioned, however, that it is not necessary to employ the intracerebral technique with the above mentioned viruses, since both may be propagated by the yolk sac route. It is interesting to note that in the chick embryo rabies behaves as a pantropic virus, with all tissues including the blood containing demonstrable amounts of the infectious agent.³² Taylor recently found it necessary to employ the intracerebral technique to *initiate* growth of Anopheles B virus, after which the yolk sac route could be used for further serial passage.²⁴ However, the Wyeomyia virus could be maintained only by brain-to-brain passages with the intracerebral technique.²⁴

Intra-ocular Inoculation. This method was used by Dawson³¹ in some of his rabies cultivation studies. It has found no widespread use.

Intravenous Inoculation. This method likewise has found no widespread use or application. However, it has value in studies concerned with the dissemination of disease producing agents upon introduction into the bloodstream and the course of the infectious process under these conditions. The method has been used for studying the distribution and persistence of immune bodies following intravenous injection³³ and the effect of specific antibodies on the course of infection.³⁴

Embryo Stab. The embryo can be inoculated, either by the usual type of approach for amniotic inoculation, the needle being injected into the embryo under direct observation, or by a jab with a long needle into the center of the egg using transillumination.³³⁻³⁵ This method may be used if desired to introduce such viruses as yellow fever, Japanese B and the equine encephalitis viruses.

Factors Influencing Growth

TABLE 2 lists the factors that influence the growth of viruses and rickettsiae in chick embryos.

The optimal conditions concerning the age of embryo, route of inoculation, dilution and volume of inoculum, temperature of incubation, and time of incubation following inoculation must be carefully determined for every viral and rickettsial agent since each is a separate and distinct problem. Space does not permit a review of the extensive work recorded pertaining to these important factors, but the interested reader is referred to the excellent review of Beveridge and Burnet.¹⁸

Thus far, relatively little work has been done to determine the effects of vitamin deficiencies on the growth of viruses. However, Mason and his associates³⁶ reported success in cultivating the virus of blue-tongue of sheep in eggs inoculated by the yolk sac route, provided the eggs were derived from fowls on a riboflavin deficient diet. Eggs from fowls on a satisfactory riboflavin diet gave no growth.

Greiff, Pinkerton and Moragues³⁷ tested a number of agents for their effect on the multiplication of typhus rickettsiae in the yolk sac and found para-amino-benzoic acid to have a rickettsiostatic activity. These results were confirmed independently by Snyder and his colleagues.³⁸ The three antibiotics, aureomycin, terramycin and chloramphenicol, have been shown to have a marked rickettsiostatic action, far superior to that of para-amino-benzoic acid, upon the growth of typhus, Q fever and other rickettsiae in the yolk sac.^{23, 39-41} Quite recently, Greiff and Pinkerton⁴² reported the introduction of antibiotic mash containing aureomycin and terramycin into the diet of a flock of laying hens resulted in progressively increased resistance of the eggs to rickettsial growth, with the resistance becoming almost complete within three weeks. Under the same conditions, the growth of influenza A virus remained unimpaired. If this report is confirmed, then obviously the work of the experimental rickettsiologist will become more complex and difficult to evaluate. Greiff and his associates³⁷ have also reported that sodium fluoride may accelerate the growth of rickettsiae in the yolk sac and have suggested that this agent may be used to render the entodermal cells of the yolk sac more susceptible to infection by certain disease producing agents.

Various chemical substances, such as nitroacridines,⁴³⁻⁴⁶ hexamidine⁴⁷ and certain aromatic diamidines⁴⁸ have been shown to inhibit the growth of influenza virus,^{45, 47, 48} mumps virus^{46, 48} and agents of the psittacosis-lymphogranuloma group^{43, 44, 46, 48} in the allantoic sac of chick embryos. Apple pectin inhibits the growth of influenza A virus (PR8 strain)⁴⁹ and polysaccharides derived from type-specific Friedländer bacilli inhibit the multiplication of mumps virus in the allantoic sac of the chick embryo.⁵⁰ Other studies on chemical inhibition of viruses by a variety of agents have been reviewed recently.⁵¹ Cortisone acetate, on the other hand, has been reported to increase the concentration of influenza (Lee and PR8) or mumps virus in the fluid of eggs.⁵²

Cultivation of Certain Viruses and Rickettsiae

TABLE 3 lists a number of virus agents that multiply readily and produce easily visible foci on the C-A membrane. TABLE 4 shows certain viruses that produce only weak foci or require adaptation to produce definite foci, while TABLE 5 lists a number of viruses that multiply on the C-A membrane without causing lesions.

TABLES 6, 7 and 8 record some of those viruses and rickettsiae which have been found to grow readily in the allantoic sac, in the amniotic sac, and in the yolk sac.

Preparation of Vaccines

TABLE 9 shows a number of viruses and rickettsiae in which the living agent, as cultivated in chick embryo tissue, is used as a vaccine for immunization purposes. In this connection, it is important to call attention to the fact that thus far there is no well-authenticated report that chick embryo tissue is a natural carrier of any virus infection. This fact cannot be over-emphasized from the safety standpoint, since an ever-increasing number of vaccines are being prepared from infected chick embryo tissues as living, modified virus agents and certainly all evidence indicates that the number

TABLE 3

VIRUSES WHICH MULTIPLY READILY ON THE C-A MEMBRANE AND PRODUCE EASILY VISIBLE FOCI*

Fowl Pox and other bird poxes	Sabin's B virus
Infectious laryngo-tracheitis	Pseudorabies
Newcastle disease	Myxomatosis
Smallpox	Ectromelia of mice
Vaccinia	Vesicular stomatitis
Herpes simplex	Pacheco's parrot disease

TABLE 4

VIRUSES WHICH MULTIPLY ON THE C-A MEMBRANE BUT WHICH PRODUCE ONLY WEAK FOCI OR REQUIRE ADAPTATION TO PRODUCE DEFINITE FOCI

Influenza A and B	Yellow Fever
Psittacosis	St. Louis Encephalitis
Lymphogranuloma Venereum	Rift Valley Fever
Louping Ill	Japanese B Encephalitis
	Canine Distemper

* Most of the agents mentioned above give well-organized lesions on the C-A membrane.

TABLE 5

VIRUSES WHICH MULTIPLY ON C-A MEMBRANE WITHOUT CAUSING LESIONS

Rabies
Lymphocytic choriomeningitis
Measles
Infectious bronchitis
Blue comb disease

TABLE 6

VIRUSES WHICH MULTIPLY READILY IN ALLANTOIC SAC

Influenza A and B (D phase)
Swine influenza
Neurotropic influenza
Psittacosis—LGV group
Fowl plague
Newcastle disease
Epidemic keratoconjunctivitis
Mumps

of such preparations will continue to grow. As Beveridge and Burnet have pointed out,¹⁸ "virus cultivation in the chick embryo much more closely resembles test tube culture of bacteria than it does orthodox animal inoculation methods." Furthermore, it should be mentioned that while it is known that maternal antibody may be present in the yolk of the hen's egg, the embryo itself has no power to produce antibody. The presence of maternal antibody in the yolk must be considered particularly by those investigators working with common enzootic viral infections in fowls, such as fowl-pox, infectious bronchitis, Newcastle disease and infectious laryngotracheitis.

TABLE 10 lists a number of vaccines in which the viral or rickettsial agents are inactivated by chemical substances such as formalin, and hence are called killed vaccines.

TABLE 11 tabulates some of the more important factors concerned in the usual laboratory practice of handling eggs infected with various viral and

rickettsial agents. It is readily seen that each infectious agent represents an entirely separate and distinct problem and the optimal conditions for growth and yield of harvested material must be worked out systematically for each one.

A few remarks should be made about the use of dead or killed and de-embryonated eggs in virus research. Rabinowitz and associates⁵³ inoculated typhus rickettsiae (*R. prowazekii*) into the yolk sac of eggs containing embryos that had been killed by chilling on the third day of incubation. Rick-

TABLE 7

VIRUSES AND RICKETTSIAE WHICH
MULTIPLY IN AMNIOTIC SAC

Influenza A and B (O and D Phase)
Swine influenza
Neurotropic influenza
Psittacosis
Infectious laryngotracheitis
Newcastle disease
Mumps
Rickettsialpox
Infectious hepatitis

TABLE 8

VIRUSES AND RICKETTSIAE WHICH MULTIPLY
IN YOLK SAC

All rickettsiae (Spotted Fever, Typhus, Q fever and Scrub Typhus Groups)	Yellow fever
Psittacosis—LGV Group	Ntaya
Equine encephalitides	Bwamba
Newcastle disease	Bunyamwera
Rabies	Uganda S
Japanese B encephalitis	Zika
Canine distemper	Haemagogus A
Colorado tick fever	Haemagogus B
Semliki forest virus	Leucocelaenus
West Nile virus	Sabethes
Canine Hepatitis	Mengo
	Anopheles A
	Ilheus
	Dengue

TABLE 9

LIVE VIRAL AND RICKETTSIAL VACCINES

Fowl Pox*	Canine distemper*
Infectious laryngo-tracheitis*	Vaccinia*
Pigeon pox*	Influenza
Newcastle disease*	Colorado tick fever
Yellow fever*	Dengue
Rabies*	Rinderpest
	Epidemic typhus

TABLE 10

KILLED VIRAL AND RICKETTSIAL VACCINES

Influenza
Mumps
Japanese B encephalitis
Equine encephalitides
Rocky mountain spotted fever
Epidemic and murine typhus
Q Fever*
Boutonneuse fever*
Psittacosis*

* Used extensively.

* Not used extensively.

ettsiae were found to multiply abundantly at 37°C. for approximately 16 days with living cells still present in the yolk sac tissue.

Lahelle and Horsfall⁵⁴ found chick embryos that had been killed by storage at room temperature for seven to ten days, or by storage at 4°C. for four days, were capable of supporting the growth of influenza virus upon further incubation at 35°C. for four to eleven days. Embryos that had been frozen at -30°C. for 20 hours did not support virus multiplication under similar conditions.

It is appropriate to mention at this time that the author,⁵⁵ in 1942, found that a combination of living and dead embryos gave an increased yield of spotted fever infected tissues (*R. rickettsii*). This procedure has been fol-

lowed since in producing vaccines. The procedure is to inoculate seven-day old embryos in the yolk sac and to incubate them at 32° to 36°C. The embryos die within three to four days, at which time numerous rickettsiae are found in the yolk sac (approximately 10^6 infecting doses per gram), but relatively few rickettsiae are found in the C-A membrane. However, if the eggs are then kept at room temperature (20° to 22°C.) for an additional two to four days, rickettsiae continue to grow, particularly in the C-A membrane,

TABLE 11
SUMMARY OF VACCINE PRODUCTION IN CHICK EMBRYOS

Infectious agent	Age of embryo (days)	Route of inoculation	Inoculum			Incubation		Harvested material			
			Volume (cc.)	Dilution	Source	Temperature (Cent.)	Time	Embryo	CAM*	CAF†	Yolk sac
Boutonneuse fever	7	Y.S.‡	0.2	10^{-2}	Y.S.	32	3-5 days		×		×
						22	2-4 days				
Canine Distemper	7	Y.S.	0.4	200%	CAM	37	7 days	×	×		×
Eastern Equine	12	A.S.§	0.2	10^{-2}	CAM	37	18 hrs.	×	×	×	
Epidemic Typhus	7	Y.S.	0.2	10^{-4}	Y.S.	36	5-10 days				×
Fowl Pox	9	A.S.	0.2	10%	CAM	37	3-4 days	×	×	×	
Influenza	11	A.S.	0.2	10^{-4} - 10^{-5}	CAF	36	48 hrs.			×	
Japanese B	9	Y.S.	0.2	10^{-3}	Y.S.	37	64 hrs.	×	×	×	
Laryngotracheitis	9	A.S.	0.2	10%	CAM	37	3-4 days	×	×	×	
Louping Ill	9	Y.S.	0.2	10%	Emb.	37	3 days	×	×	×	
Mumps	8	A.S.	0.2	10^{-3}	CAF	36	6 days			×	
Murine Typhus	7	Y.S.	0.2	10^{-4}	Y.S.	36	5-10 days				×
Newcastle Disease	11	A.S.	0.2	10^{-4}	CAF	36	90 hrs.	×	×	×	
Pigeon Pox	12	CAM	0.2	10%	CAM	37	5 days		×		
Psittacosis-LGV	9	Y.S.	0.2	10^{-2}	Y.S.	36	6-7 days				×
Q Fever	7	Y.S.	0.2	10^{-4}	Y.S.	36	6-10 days				×
Rabies	7	Y.S.	0.2	10^{-2}	Emb.	36	8-10 days	×			
Rocky Mt. Spotted Fever	7	Y.S.	0.2	1:50	Y.S.	32	3-4 days		×		×
						22	2-4 days				
Vaccinia	11	CAM	0.25	10^{-5}	CAM	37	3 days		×		
Venezuelan Equine	12	A.S.	0.2	10^{-2}	CAM	37	18-24 hrs.	×	×	×	
Western Equine	12	A.S.	0.2	10^{-2}	CAM	37	24-30 hrs.	×	×	×	
Yellow Fever	7	Y.S.	0.06	10^{-4}	Emb.	37	4 days	×			

* CAM = Chorio-allantoic membrane. † CAF = Chorio-allantoic fluid. ‡ Y.S. = Yolk sac.

§ A. S. Allantoic sac.

so that this tissue shows a growth approximately equivalent to that found in the yolk sac. The increased growth in the C-A membrane at room temperature has been confirmed by both microscopic examination and quantitative complement-fixation tests using infected C-A tissues as antigen. Both the C-A and the yolk sac membranes are then harvested for vaccine or antigen production. All rickettsiae of the spotted fever group behave similarly in this respect, whereas those of the typhus, Q fever and scrub typhus groups do not.

Bernkopf⁵⁶ cultivated influenza virus in the C-A membrane of de-embryonated eggs. This procedure allows the introduction of a greater volume of fluid, makes possible the use of a synthetic medium free of urates, allows

for a more accurate study of virus multiplication, and is useful in the study of inhibitors and activators.

Finally, reference must be made to the extremely informative and valuable studies carried out through use of the chick embryo to determine the cycle of growth, morphological forms and interference and recombination phenomena, particularly with influenza virus. For these studies the reader is referred to the papers of the Henles,⁵⁷⁻⁵⁹ Hoyle,⁶⁰ Isaacs,^{61, 62} Chu,⁶³ von Magnus⁶⁴ and Burnet.²¹

In conclusion, I have tried to show how extremely valuable and versatile the chick embryo is for viral and rickettsial research and production problems, and how practically all of the known viral and rickettsial agents can be propagated in the tissues of the developing chick embryo. Recently, two of the more important virus agents in the field of human and veterinary medicine, namely those of dengue fever and foot and mouth disease, have been added to the list of those viruses cultivated in embryonic chick tissues.^{65, 66} In addition, cultivation of the virus of canine hepatitis has been reported⁶⁷ but this latter work has not yet been confirmed. Hog cholera and poliomyelitis viruses are notable exceptions in that they have thus far resisted all attempts to propagate in chick embryo tissues, but I am confident that some one will eventually succeed in adapting even those most difficult ones. As we all know, success is a combination of luck and perseverance (perhaps perspiration should be included too), and the one who is most likely to succeed is the one who is most unwilling to take "no" for an answer.

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Discussion

DR. DAVID A. KARNOFSKY (*Sloan Kettering Institute for Cancer Research, New York, N. Y.*: Dr. Cox has noted that fowl leucosis virus will grow in the egg but has not commented upon the Rous sarcoma virus. It should be stated that while the fowl leucosis virus may be transmitted through the egg, only living leucosis cells inoculated into the egg are capable of producing active leucosis in the chick embryo. On the other hand, the Rous sarcoma virus inoculated onto the chorio-allantoic membrane of the chick embryo will produce proliferative lesions as well as a hemorrhagic disease of the embryo.

THE PATHOLOGICAL EFFECTS OF VIRUSES ON THE CHICK EMBRYO

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The chick embryo becomes diseased when inoculated with any of the viruses to which it has been found to be susceptible. Pathological effects, attributable to the infectious process, are produced and provide a unique opportunity for the study and comparison of the pathology and pathogenesis of viral infections.¹⁻³ The true identity of a particular virus perhaps is realized only within the confines of the susceptible cell. Its behavior as a disease-producing agent cannot be completely characterized by innate attributes but may be derived from those which characterize a virus as parasite within the parasitized host cell. Neither virus nor susceptible cell alone, but the intracellular processes of infection are basic in the consideration of viral diseases. The infected chick embryo provides a ready means for observing the effects of these processes.

A systematic study and comparison of viral infections of the chick embryo, performed under uniform conditions of technical procedures and experimental control, still remains to be undertaken. The investigations which have been published regarding the pathological effects of virus infections of the embryo have varied considerably in respect to experimental procedures and technical detail. Although, strict comparisons cannot be drawn for these reasons, a review of a selected series of these studies should serve to emphasize the importance of the pathological approach to the study of the nature of viral diseases.

The intracellular processes of infection which characterize viral infections have come to be recognized in the form of changes which take place in the parasitized cells. These include hyperplasia and hypertrophy, the development of inclusion bodies, swelling degeneration, and lysis and necrosis. These changes take place especially within those host cells for which particular viruses have an enhanced predilection. These specific cytotropisms often serve to identify viruses, and the character of the changes induced has come to be accepted as pathognomonic.

The more specific effects of various virus infections are especially likely to develop at a definite stage of embryonic development. Embryos of 10- to 14-day incubation are not only highly susceptible but the lesions and reactions which develop in many instances closely simulate those observed in the natural or experimental host. Possible reasons for this more specific response to infection during this stage are discussed elsewhere.⁴ Most of the investigations on the pathology of viral infections of the embryo have been made during this stage of development. Inoculation of the chorio-allantoic membrane has been the usual route of infection initiation. In certain instances, intra-amniotic, intra-embryonic or intra-cerebral inoculations have been used for special reasons or for special effects.

The hyperplasia and hypertrophy of cells which characterizes the response

of susceptible cells to infection with certain viruses is well exemplified by infection of the chorio-allantois of 10- to 14-day-old embryos with the virus of fowl pox.⁵ The membranal lesion essentially consists of a pock-like eruption. The ectodermal epithelium is most profoundly affected. Extensive hyperplasia and hypertrophy of these cells occurs with relatively little tendency of this process to progress to necrosis. Nearly every cell in involved areas is parasitized by virus as evidenced by the presence of typical intracytoplasmic inclusions in each cell. Cells of the entodermal layer are less extensively affected and those of the mesoderm are spared.

A variant of fowl pox virus induced by intracerebral inoculation of newly-hatched chicks possesses affinity for mesodermal cells as well as those of the membranal ectoderm and endoderm.⁶ Parasitized ectodermal and endodermal cells undergo rapid necrosis instead of hyperplasia and hypertrophy. Vascular endothelial cells and fibroblasts in the mesoderm containing typical inclusions are present in great numbers.

Lesions in the embryo itself which would indicate spread of the virus by way of the circulation from the infected chorio-allantois occur with great infrequency and embryos rarely die from membranal infections.⁵ It has been possible, nevertheless, to demonstrate that virus is present in the circulation in small amounts. Direct inoculation of the embryonic skin produces typical epidermal lesions.

The effects of infection on the embryo with vaccinia virus are much more profound. In most instances, the majority of 12- to 14-day embryos die within 72 to 96 hours following inoculation of the chorio-allantois.⁷ The membranal lesion is essentially a pock-like eruption. The initial point of attack by the virus is upon the ectodermal epithelium. Focal proliferations develop in which the cells become swollen and vacuolated within 24 hours after inoculation. In many of these, parasitization by the virus is evident from the presence of intracytoplasmic inclusions. Destruction of the affected cells is rapidly effected so that ulceration of the membranal surface ensues. An acute inflammatory reaction develops within the mesodermal layer in response to the necrosis of ectodermal cells.

There is marked variation in the capacity of different strains of vaccinia virus to infect cells of the mesodermal and endodermal layer of the chorio-allantois. So-called neurotesticular strains retain their marked capacity to infect mesodermal and endodermal cells through numerous successive membranal passages.⁸ These strains, more than any others, invade vascular endothelium destroying capillary and venule walls, and produce characteristic hemorrhagic lesions. Different strains of vaccine virus used commercially for the production of smallpox vaccine vary greatly in their capacity to infect cells of the mesoderm and endoderm of the membrane.⁹ Chick-embryo-membrane-adapted strains gradually become more and more ectodermotropic upon continued passage.¹⁰

Vaccinia virus has a marked capacity for spreading to the embryo from the membranal lesion by way of the blood stream.¹¹ Embryos which survive more than four days following membranal inoculation develop a generalized disease. Focal necrotic lesions are observed in most tissues and organs

except the central nervous system. A typical pock-like exanthem involves the skin and the mucous membranes of the pharynx. The evolution of these lesions is of particular interest in exemplifying the effect of vaccinia virus on dermal structures in which it induces its classical lesion. Under these circumstances the development of the pock from the earliest involvement of cells in the basal layers of the epithelium, with subsequent hyperplasia and hypertrophy followed by central necrosis and eventual ulceration, can be observed from stage to stage. Throughout this process the parasitization of cells can be ascertained by the presence of typical intracytoplasmic inclusions.

Downie¹² has described the effects of infection of the chorio-allantois with the virus of naturally-occurring cow-pox. In most respects this process resembles that observed with vaccinia, with the notable exception that the inclusion bodies in cow-pox are generally larger, more homogenous in appearance, and more eosinophilic in their staining reaction. The effect of this infection on the embryo itself was not studied in detail.

Variola virus is readily established in the chorio-allantois of the 10- to 14-day-old embryo.¹³⁻¹⁶ Strains of the virus responsible for the milder clinical forms of smallpox have been studied more extensively than those producing the severe form of the disease. Variola apparently restricts its effect to the inoculated chorio-allantois to a much greater degree than does vaccinia. A characteristic pock-like eruption develops on the chorio-allantois within 48 to 72 hours following inoculation. These pocks are composed of focal proliferations of ectodermal epithelial cells. Parasitized cells contain typical cytoplasmic inclusions. Necrosis of cells at the center of the pocks produces ulceration. A mild inflammatory reaction involves the underlying mesoderm. No invasion of mesodermal or endodermal cells has been observed. The infection remains superficial and invasion of the embryo has not been described. It is evident from Smadel's observation¹⁷ that strains exist which have the capacity to invade the embryo from the membrane. The strain which Smadel isolated regularly kills embryos within four to five days after membranal inoculation. Descriptions of the embryonic pathology under these circumstances have not been published.

Herpes simplex virus infects the chorio-allantois and readily generalizes to the embryo.^{18, 19} The membranal lesion also consists of a pock-like eruption which develops in 48 to 72 hours following inoculation. The focal lesions comprise proliferations of ectodermal epithelial cells, many of which exhibit typical type A intranuclear inclusion bodies. Parasitized cells are rapidly destroyed, and widespread ulceration of the infected membrane is commonly observed. Anderson's extensive studies have demonstrated that herpes simplex readily spreads by way of the blood stream from the membranal lesion to the embryo. In early passages, encephalitis develops with marked involvement of the central nervous system with focal areas of neuronal destruction. This effect was not observed beyond the third passage of the H. F. strain of the virus. Other tissues and organs are more commonly affected after adaptation of the virus by passage. Metastatic focal lesions consisting of areas of central necrosis, surrounded by a zone of parasitized

cells containing intranuclear inclusions, are observed in the heart, liver, spleen, kidneys, and striated muscle as well as involving the vascular endothelium in various localities. Introduction of tenth passage virus into the amniotic sac resulted in widespread infection of the skin and the mucous membranes of the mouth and pharynx. These were characterized as focal proliferative cutaneous and mucous membrane lesions in which cells containing herpetic inclusions were abundant. Direct inoculation of embryonic skin induced local cutaneous papules from which site the infection spread to the underlying subcutaneous tissue, the striated muscle and peritoneum. In one instance, subcutaneous inoculation initiated an ascending destructive myelitis of the spinal cord. Intracranial inoculation invariably produced a destructive encephalitis.

Rabies virus was first successfully adapted to propagation in the embryo by direct intracerebral inoculation.¹⁹ This induced a grossly visible symmetrical hydrocephalus. Ten- to 14-day-old embryos were found to be most useful. Inoculated embryos survived at least six to eight days although many were found to survive considerably longer, up to 13 or 14 days without hatching. Besides the hydrocephalus, the effect of rabies virus on the embryonic brain consists of hydromyelia, encephalomalacia and myelomalacia of great extent and marked degree. Inflammatory reaction to the destructive process is mild, consisting chiefly of large mononuclears within the central nervous system. Outside the central nervous system, large collections of lymphocytes surround affected dorsal root ganglia, sympathetic ganglia and the intrinsic ganglia of the various viscera.

The specific response to infection with rabies virus consists of the presence of typical Negri bodies, chiefly within the cytoplasm of neurons. They are observed to be uncommonly numerous in the infected chick embryo, especially six to seven days following inoculation. The neurons of the central nervous system are predominantly involved, but Negri bodies are also present within ependymal epithelium, retinal cells and within neurons of the peripheral ganglia.

In the original experiments, rabies virus appeared to remain restricted in its infectivity for nervous tissue. Since then, strains have been adapted to the embryo which appear to have developed considerable pantropism.²⁰ Adequate descriptions of the effect of the virus under these circumstances have not been published.

Discussion

It is not possible within the limits of the available time to review all the published observations on the effect of different viruses on the chick embryo. The examples chosen, perhaps more than any others, represent attempts at a reasonably complete study of the pathology of these specific virus diseases induced during a definite stage of embryonic development. They illustrate the great potentialities which are inherent in a careful histo-pathological study of these phenomena. As they stand, however, they only afford brief glimpses into the behavior of viruses, or more specifically, what, by means of the histological method, can be observed to happen to cells parasitized by

these particular viruses when embryos are infected during the tenth to 14th day of incubation. They support the concept of cytotropism, which is basic to an understanding of diseases of viral etiology. They do not provide information, however, from which inferences may be drawn regarding the why or how of cytotropism.

The susceptible cells provide the substrate of biophysical and biochemical reactions systems which are required for virus multiplication. These metabolic activities which support, or, perhaps more correctly, become involved in, the genesis of viruses characterize the susceptibility of cells to virus infection. The chick embryo is susceptible to infection during successive stages of development. Certain types of metabolic processes are predominant at each of these stages. Specific enzyme and hormonal activities manifest themselves at definite periods of development and cellular and tissue differentiations are accomplished at well-recognized intervals of incubation. Susceptibility to virus infection is manifest during each of these stages. The effects which are produced under these different conditions in which viruses appear to thrive are markedly different in each instance. It has been shown, for example, that infection with Newcastle virus can be established in the first 24 to 48 hours of embryonic development with the effect of producing recognizable malformations in the otic and optic structures.²¹ Viruses proliferate with great rapidity and in prodigious numbers in embryos of six to nine days incubation. Under these circumstances, they do not manifest the more specific cellular affinities which characterize their behavior in infection of embryos in the 10- to 15-day stage. Beyond the 15-day stage to hatching, susceptibility of cells becomes more restricted to those more specific cytotropisms which characterize infection of the adult natural host. These sharp differences in behavior of viruses and their effect on susceptible cells which compose the organs and tissues of the embryo can be subjected to a more critical analysis and comparison than has been undertaken.

A systematic study and comparison of the effects of infection on the embryo with a particular virus during its successive stages of development, under uniform conditions of dosage, route of inoculation, temperature of incubation and other readily controllable conditions, should discover important data concerning the nature of the host cell-parasite relationships, from which the manifestations of virus diseases proceed. Its ultimate solution undoubtedly will be derived from the discovery of the mechanisms whereby intra-cellular metabolic processes are diverted to the support of the parasite. These problems more directly concern the biochemist and biophysicist. Certain encouraging beginnings in this direction have been made recently. The traditional disciplines of histogenesis and pathology will prove indispensable in these efforts. The basic data supplied by the accurate description of the effected morphological changes will be required for an adequate interpretation of the phenomena observed in the infectious processes induced by viruses. This will require organized cooperative effort. This monograph, in which the broad general biological approach has been attempted, points the way toward the eventual solution of the problems presented in this discussion.

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THE METABOLIC INTERACTIONS OF INTRACELLULAR PARASITES AND EMBRYONATE EGGS

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A high degree of coordination must exist within cells in order to permit an orderly progression of the metabolic processes necessary for cellular activity, growth and differentiation. Cellular structural components and enzymes are linked in unknown integrated arrangements of biochemical units. The metabolic processes which lead to cell maturation are complex and involve a multiplicity of extrinsic factors and intrinsic mechanisms. Within this cellular milieu viruses and rickettsiae live, grow and reproduce.

Most investigators hold that viruses and rickettsiae require complementary enzyme systems.^{14, 37} Thus the host-cell supplies missing enzymes or perhaps biochemical intermediates necessary for continued enzymatic activity. Numerous biochemical studies made on the developing chick embryo have shown that tissues, organs and cells undergo many changes in biochemical composition as they develop towards their adult functional state.^{3, 31} It has been shown, for example, that differentiation of the developing chick embryo toward final adult form is characterized by a rising protein content of embryonic cells. The dominance of anabolism over catabolism in growing cells must be due not only to the increased formation of enzymes but also to the changes in regulating mechanisms, which not only modify reaction rates but change the direction of reactions. The changes during development must be the basis for the observations that rickettsiae multiply best in the yolk sac of six- to eight-day-old embryos, the influenza virus in the allantoic cavity of ten- to 11-day embryos, or vaccinia virus in the chorio-allantois of 12- to 14-day embryos.⁵

Structural alterations of the developing embryo have been produced by vitamin deficiencies, Xrays, injection of chemical compounds, virus infection and transplantation of tumor tissue.^{11, 22, 26, 28, 47, 50} The changes in metabolic pathways of the developing embryo brought about by the foregoing agents are still in the realm of speculation. The present report is concerned with our attempts to explore this realm through quantitative measurements of oxygen consumption and growth of developing chick embryos under experimental conditions. Work done in this laboratory and elsewhere has shown that artificially produced modifications in embryonic respiration alter the growth rate of rickettsiae, and, conversely, that the presence of multiplying rickettsiae and Influenza A virus alters the respiration of the embryos.^{16, 17, 18, 19, 20, 21, 24, 26, 29}

The term "growth" is often used to denote the sum total of processes by which protoplasmic mass increases in size and complexity. Different investigators divide the process of protoplasmic increase into different component parts. The division proposed by Needham³² with slight modifications by Brody⁴ is most widely held. The latter worker proposed that growth be treated as a mechanism of biologic synthesis, *i.e.*, the biological

manufacture of new biochemical units. This phenomenon is concerned with one or all of three processes: (1) cell multiplication, (2) cell enlargement, and (3) incorporation of environmental materials.

Recent investigations employing isotopes have shown that many cellular secretions, such as bone matrix, plasma, *etc.*, although qualitatively and quantitatively constant within the body, are in a state of rapid flux between storage depots and the cells of the organism.⁴⁸ Thus it would appear that Brody's third growth process is vital to the proper functioning of cell multiplication and enlargement.

The basic data for growth studies were obtained by dehydrating embryos, after removing extra-embryonic membranes, to constant weights in an oven maintained at 105°C. Preliminary studies showed a high degree of correlation between dry embryo weight and wet embryo weight. The former, however, showed less variation, as measured by the standard deviation of the sample. The statistical significance of differences between mean dry weights of groups of embryos was determined by "student's" test.²⁵

Oxygen consumption was measured by the method recently described by Greiff and Pinkerton.¹⁹ Groups of 15 to 20 eggs were placed in air-tight containers. These containers were immersed in a constant temperature water bath maintained at the same temperature as that used for previous incubation. After reaching temperature equilibrium a ten cc. sample of air was removed by means of a greased syringe. A second sample of air was removed one hour later. Both samples were analyzed for oxygen content, using a Haldane-Henderson gas analyzer. Knowing the volume of the containers, the volume and number of eggs, the oxygen percentage of each sample, and the time elapsed, it was possible to determine the amount of oxygen consumed per egg per hour. Differences in the oxygen consumption between groups of eggs were evaluated for statistical significance by the method previously developed.¹⁹

Pinkerton and Hass,^{40, 41} Zinsser and Schoenbach,⁵⁴ and Pinkerton and Bessey³⁸ have shown that increased rickettsial growth was correlated with environmental factors thought to decrease the metabolism of the host cell. Greiff and Pinkerton¹⁷⁻²¹ showed that toluidine blue, increased temperature (40°C.), para-aminobenzoic acid and penicillin increased oxygen consumption and were rickettsiostatic. Lowering the temperature of incubation (37.5°C.) and potassium cyanide were found to decrease oxygen uptake and increase the growth of rickettsiae.

Although rickettsiae grow very sparsely at 40°C. under normal conditions, they multiply freely if KCN is added in amounts sufficient to lower oxygen consumption, but insufficient to cause embryonic death. Thus there appeared to be an inverse relationship between rickettsial growth and oxygen consumption. The multiplication of typhus rickettsiae in embryonate eggs caused an initial increase in oxygen consumption, followed by a prolonged decrease (TABLE 1).

During the course of the foregoing work, it was noted that the embryos of eggs incubated at 40°C appeared to be of larger size than those maintained at 37.5°C. The embryos of eggs inoculated with rickettsiae, on the

other hand, appeared smaller than uninfected embryos of the same age. The following investigation was undertaken to determine if increased temperature and rickettsial infection influenced oxygen uptake primarily by influencing respiratory enzymes or by accelerating and retarding embryonic growth.

Preliminary experiments showed that in uninfected eggs the curves for both oxygen consumption and embryo dry weight were approximately linear between the eighth and thirteenth day of embryonic life (TABLES 2 and 3). Therefore, for ease and reliability of calculations, these ages were selected. Following eight days of incubation at 37.5°C, oxygen uptake and embryo

TABLE 1
THE EFFECT OF INFECTION WITH TYPHUS RICKETTSIAE ON OXYGEN CONSUMPTION OF EMBRYONATE EGGS

Age of embryos (days)	Oxygen consumption per egg per hour (cc. O ₂ /egg/hr.)		Degree of infection†
	Uninfected eggs	Infected eggs	
6	1.23	1.18	0*, 0*, 0*, 0, 0
6.5		Rickettsiae injected	
7	1.56	1.27	0*, 0*, 0*, 0*, 0, 0
9	2.08	2.16	0, 0, 0*, 0*, (+)*
11	4.65	5.11	0*, 0*, 0, (+), (+)
12	7.22	5.49	(+)*, 1*, 1, 1*, 2*
13	8.95	6.34	2*, 3*, 3, 4, 4
14	10.83	7.11	4, 4, 5, 5, 5
16	14.26	2.60	4, 5, 5, 5, 5

* Embryo alive at time of examination.

† Each figure represents an individual egg.

0 No rickettsiae recognized.

(+) Less than one rickettsiae per oil immersion field.

1 1-10 rickettsiae per oil immersion field.

2 10-100 " " " " " "

3 100-1000 " " " " " "

4 1000-5000 " " " " " "

5 5000-10000 " " " " " "

dry weight were determined for a group of 20 eggs. Other similarly incubated eggs were divided into groups of 20 and transferred to incubators maintained at various temperatures. Several investigators^{35, 43, 44} have shown that the developing chick embryo is poikilothermic up to 14 days of incubation. The temperatures of the developing chick embryos were dependent, therefore, upon the environmental temperatures. After five days of incubation (13 days of embryonic life), oxygen consumption and embryo dry weight were again determined. The total oxygen consumption was calculated by integrating the area under the straight line curve connecting the oxygen uptake of the eighth and 13th days. Dividing the total oxygen consumed by the total weight gained between the selected ages gave the oxygen uptake per milligram gain in dry weight. The results obtained are shown in TABLE 4.

The mean embryo dry weights and the temperature of incubation were

found to bear a direct relationship to one another. The increases in mean embryo dry weights were not, however, directly proportional to the elevation of temperature. Raising the temperature 3.5°C from 34 to 37.5°C resulted in an increased mean dry weight of 301 mg. while the 3.5°C temperature increment from 37.5 to 41°C resulted in an increased mean dry weight of 101

TABLE 2

OXYGEN CONSUMPTION OF EMBRYONATE EGGS INCUBATED AT 37.5°C.

Age of embryos (days)	Oxygen uptake (cc. O ₂ /egg/hr.)
5	.41
6	.65
7	.83
8	1.43
9	2.61
10	4.00
11	5.47
12	6.90
13	9.05
14	11.36
15	13.88

TABLE 3

MEAN EMBRYO DRY WEIGHTS OF EGGS INCUBATED AT 37.5°C.

Age of embryos (days)	Mean dry weight (mg.)
5	15 ± 2
6	31 ± 7
7	50 ± 8
8	73 ± 13
9	110 ± 10
10	280 ± 47
11	484 ± 43
12	726 ± 66
13	955 ± 53
14	1,147 ± 58
15	1,501 ± 79

TABLE 4

THE EFFECT OF TEMPERATURE ON TOTAL OXYGEN UPTAKE AND DRY WEIGHT INCREASE OF EMBRYONATE EGGS FROM EIGHT* TO THIRTEEN DAYS OF AGE

Temperature of incubation (°C.)	Mean dry weight† (mg.)	Oxygen consumption† (cc.O ₂ /egg/hr.)	Total oxygen uptake (cc.)	Dry weight increase (mg.)	cc. O ₂ /mg. gain dry wt.
34	347 ± 14	5.3	313.8	285	1.09
36	546 ± 21	8.7	525.0	484	1.08
37.5	659 ± 18	10.8	648.2	597	1.08
39	705 ± 15	11.5	691.0	643	1.07
40	721 ± 19	10.8	648.4	659	.98
41	733 ± 20	7.5	447.1	671	0.66

* Eight-day-old embryos, incubated at 37.5°C., used 0.31 cc. of oxygen per hour. The mean embryo dry weight of this group 62 ± 4 mg.

† Thirteen-day-old embryos.

mg. Similar findings have been reported by Henderson and Brody,²⁴ Henderson,²³ and Romanoff *et al.*⁴⁵ Rodinini⁴⁶ has also shown that the incubation of embryonate eggs at elevated temperatures (40–42°C) causes a decrease in the growth rate of the chick embryo.

The ratios of oxygen consumption to gain in dry embryo weight varied from 1.10 to .98 as the temperature of incubation was increased from 34 to 40°C. At 41°C, however, the index ratio dropped to 0.66 (TABLE 4). Crozier⁷⁻⁹ has reported similar "breaks" in metabolic activity.

Fletcher and Hopkins¹⁰ suggested that decreased O₂ consumption of the growing embryo at the higher temperature may result in an oxygen debt.

Thus at higher temperatures some metabolic processes may shift from respiratory to fermentative oxidation. With this shift, significant changes in the nature and concentration of metabolic intermediates and end-products may create an internal environment in which rickettsiae cannot survive. This shift may also limit the availability of enzymatic processes to the parasitic rickettsiae. Less specifically, as the temperature of incubation is raised, denaturation of enzymes, similar to those found in heated protein, may occur.⁶

The effect of rickettsial infection on oxygen uptake and growth was also studied. We¹⁹ have shown that the rate of increase of oxygen uptake in eggs infected with rickettsiae was significantly lower than in normal eggs. The reduced rate of oxygen consumption was found to occur several days before the degree of infection was great enough to cause the death of large numbers of embryos. In the present investigation, infection with rickettsiae was also found to decrease the growth of the embryos. Oxygen consumption and embryo dry weight were determined on a group of 20 eggs incubated at 37.5°C for eight days. A suspension of typhus rickettsiae was inoculated at this time into another group of this series. A third group acted as a uninfected control. These groups were maintained at 37.5°C for five days. Oxygen consumption and mean dry weights of the 13-day-old embryos was determined. The eight-day-old embryos consumed .45 cc. of oxygen per egg per hour. The mean embryo dry weight was 58 ± 3 mg. The 13-day-old control eggs were found to have an oxygen uptake of 9.87 cc. of oxygen/egg/hour and a mean dry weight of 501 ± 17 mg. The 13-day-old infected embryos were found to have an oxygen uptake of 6.36 cc. of oxygen/egg/hour with a mean dry weight of 461 ± 31 mg. Smears of the yolk sacs of these eggs showed approximately 100-1000 organisms per oil immersion field. The ratio of O₂ uptake per mg. gain in dry weight for the controls was 1.1 and that for the infected eggs 1.00. These values agree well with those obtained from the aforementioned temperature studies. Thus infection with rickettsiae seems to produce an orderly and correlated decrease in the enzymes necessary for growth and respiration in the embryo.

A possible mechanism for the above is suggested by the work of Woods and Dubuy⁵¹⁻⁵³ on the relationship between tobacco-mosaic virus and cell metabolism. These investigators found that nitrogen starvation brought about the reduction of a cyanide-sensitive respiratory system. This system (A-system) is in part identical with cytochrome oxidase. They also found that synthesis of virus protein proceeded at the expense of the chromoprotein (chlorophyll-protein). They postulated that the protein building units of the cell were necessary for the synthesis of the cyanide-sensitive respiratory enzyme, chromoprotein and virus protein. The first two reactions were reversible. The virus, therefore, not only used protein building units of the cell but continued growth at the expense of both the respiratory enzyme and the chromoprotein. Woods has also pointed out that the signs of tobacco mosaic infection are in part those of nitrogen starvation.

Takahashi⁴⁹ has shown that tobacco mosaic virus growth increased concurrently with host-protein breakdown. He also found that the increase

of virus protein is not dependent solely on the ability of the host cells to synthesize normal proteins. The living tobacco cell was found to be incapable of utilizing the proteins of tobacco-mosaic virus to support respiration. Thus the availability of nitrogenous compounds is a limiting factor for some of the metabolic mechanisms of the cell. The irreversible removal of cellular protein by virus, therefore, has an important effect on the anabolic processes of infected cells.

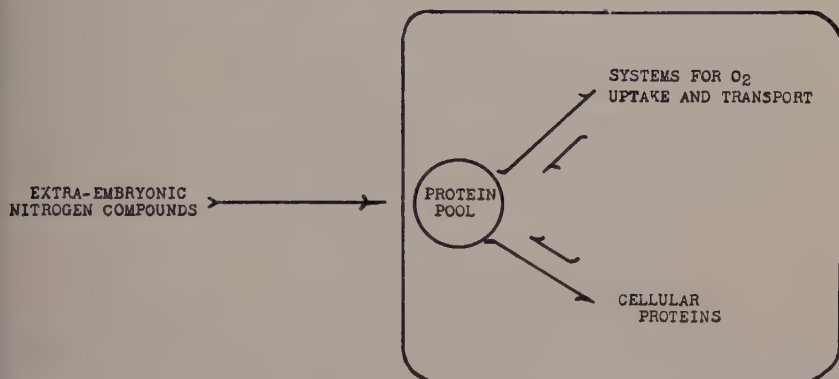


FIGURE 1. Diagram of the interaction of cellular components of normal cells of the developing chick embryo. The lengths of the arrows express assumed reaction rates.

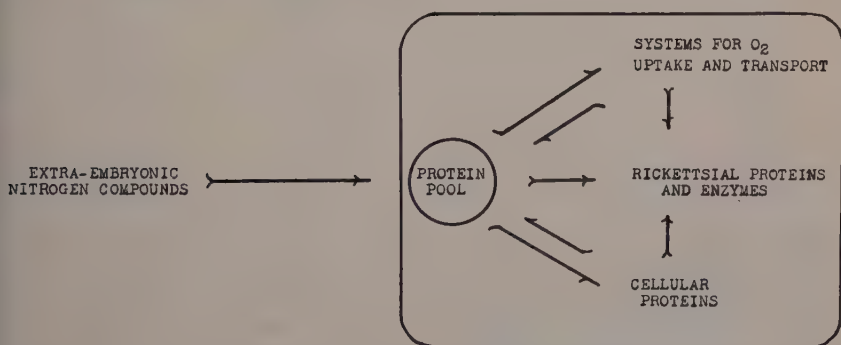


FIGURE 2. Diagram of the interaction of cellular components of infected cells of the developing chick embryo. The lengths of the arrows express assumed reaction rates.

A hypothetical interrelation of cellular components in normal and typhus infected cells, based on the findings of Woods and Dubuy and Takahashi for tobacco mosaic virus, is shown in FIGURE 1 and FIGURE 2. Transfer to the growing rickettsiae of host cell protein and of materials from the systems normally concerned with O₂ uptake and transport, as well as building blocks from the "protein pool" through direct contribution to the rickettsiae, would result in an orderly and coordinated decrease in oxygen uptake and growth of the infected cell. "Protein pool," as employed here, refers not to a mass but to a point in the dynamic equilibrium between integral cellular protein and "building blocks". The embryonate egg, however, is a rapidly

growing organism and utilizes large amounts of nitrogenous materials. The depletion of the protein pool of the infected cells, therefore, would result in a proportionally greater amount of the exogenous nitrogen compounds being transferred to these cells. This aggregation would result in a relative nitrogen starvation among the non-infected cells of the embryo with a consequent lowering of enzyme formation and reaction. These cells, therefore, would show decreased oxygen uptake and growth.

Changes in the oxygen consumption of the embryo may be demonstrated in the absence of active growth of an infective agent. We have shown that the allantoic fluid of fertile eggs infected with influenza virus contains a factor which, when injected intra-allantoically into a new series of eggs, profoundly and rapidly modifies the respiration of the embryos.¹⁶ The injection of approximately one cc. of this fluid into 11-day-old eggs resulted in a marked increase in oxygen consumption within 90 minutes. The injection of greater amounts (three cc.) of fluid caused a significant drop in

TABLE 5

THE OXYGEN CONSUMPTION OF EMBRYONATE EGGS FOLLOWING THE INJECTION OF VARYING AMOUNTS OF SALINE, NORMAL ALLANTOIC FLUID AND INFECTED ALLANTOIC FLUID

Amount of fluid injected (cc.)	Oxygen consumption (cc. O ₂ /egg/hr.)		
	Saline	Normal allantoic fluid	Infected allantoic fluid
.125	4.89	5.28	4.67
.25	4.91	4.95	5.13
.50	5.25	5.01	4.89
.75	5.16	5.33	7.55
1.00	4.83	4.97	6.81
2.00	5.18	5.11	5.84
3.00	5.00	5.26	2.93

oxygen uptake (TABLE 5). Injections into the yolk sac gave similar alterations in respiration.

The virus factor was destroyed slowly by heating infected allantoic fluid at 56°C. Infectivity of the fluid was completely lost by heating for 20 minutes. Haemagglutination disappeared after heating the fluid for ten minutes. The stimulating effect of 1.0 cc. of intra-allantoically-injected fluid was not destroyed by heating for 20 minutes, but disappeared after heating for 30 minutes. When 3.0 cc. of fluid was injected, the depressing effect on respiration was changed to a stimulating effect when the fluid had been heated between 20 and 30 minutes at 56°C (TABLE 6). Injection of fluids by the yolk sac route resulted in changes similar to the foregoing.

These results suggest that the influenza virus factor (toxin) differs from rickettsial toxins^{2, 12, 27, 33} and from the toxin produced by the lymphogranuloma venereum and Newcastle disease viruses.^{13, 42} These toxins are rapidly destroyed at 56°C and are closely associated with active rickettsiae or virus particles, while the effects of the influenza virus factor which we have studied may be separated from those of the active virus by heating.

Thus the influenza factor appears more analogous to bacterial toxins than to rickettsial toxins and lymphogranuloma venereum toxin. Whether the influenza factor will prove to be antigenic remains to be seen.

Recently we have been able to concentrate allantoic fluid by dehydration at -30°C . Infected fluids were heated at 56°C for 20 minutes to destroy virus and haemagglutinability. Normal fluids were heated similarly. Five- and ten-fold concentrations of normal allantoic fluid injected intra-allantoically neither stimulated nor depressed oxygen uptake of embryonate eggs. Five- and ten-fold concentrations of infected fluid caused a marked depression in oxygen consumption. This lowering of oxygen consumption lasted approximately 20 hours. Few deaths occurred. Studies of the effect of the virus factor on protein synthesis (growth) have not been made as yet.

In another series of experiments we made coincident determinations of infectivity and oxygen consumption at intervals during a growth period of 96 hours.³⁹ Three series of embryonate eggs were placed in environmen-

TABLE 6

OXYGEN CONSUMPTION OF EMBRYONATE EGGS FOLLOWING THE INJECTION OF ONE CC. AND THREE CC. OF NORMAL AND VIRUS INFECTED ALLANTOIC FLUIDS AFTER HEATING FOR VARIOUS TIME PERIODS

Heating period (min. 56°C)	Oxygen consumption (cc. O_2 /egg/hr.)			
	One cc. normal fluid	Three cc. normal fluid	One cc. infected fluid	Three cc. infected fluid
0	2.82	2.75	4.74	1.30
10	2.95	3.04	4.30	1.33
20	3.16	2.88	4.62	1.76
30	3.41	2.99	3.25	4.87
60	3.16	3.21	3.49	6.41

tal temperatures of 34°C , 37.5°C and 40°C immediately after the injection of virus. FIGURE 3 shows the relatively uniform increase in oxygen uptake of normal eggs. This figure also shows the effect of environmental temperature on the rate of increase.

Infected embryonate eggs showed periodic increases and decreases in the rates of oxygen uptake (FIGURE 4). At 37.5° and 40°C ., the development of infectivity titres of $10^{-5.5}$ and $10^{-4.5}$, respectively, during the first ten hours of virus growth, coincided with definite interruption of the normal increase in oxygen consumption. At all three temperatures, the oxygen uptake curves showed significant peaks 24 or 25 hours after inoculation. Corresponding peaks in infectivity titres also occurred at this time. During the next 11 or 12 hours, decreases in oxygen consumption rates were recorded, along with corresponding drops in infectivity titres. Thereafter, a relatively constant titre of infectivity was maintained at 37.5°C ., while the oxygen consumption rose to a peak at 60 hours and then fell rapidly. At 34°C ., the oxygen consumption rose between the 36th and 48th hours, and thereafter remained constant, while the infectivity titre fell somewhat

and then rose slowly. At 40°C., three distinct peaks in infectivity titre and three corresponding peaks in oxygen utilization can be noted, although the general trend in oxygen consumption was upward. The lethal effect on the embryos was greatest at 37.5°C., and the highest and best sustained infectivity titres were noted at this temperature. The lethal effect on the embryos was least marked at 40°C., and the lowest and most variable infectivity titres were found at this temperature.

It seems probable that in part the cyclic alterations in oxygen consumption

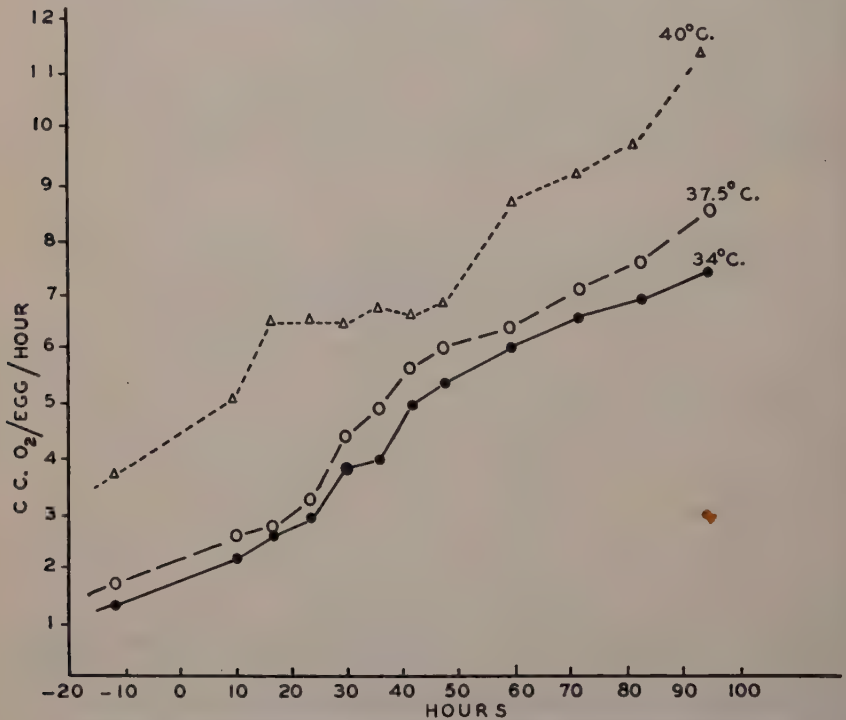


FIGURE 3. Oxygen uptake curves of normal embryonate eggs at different temperatures

and related changes in virus concentration are the result of periodic variation in the concentration of the "virus factor." During initial virus growth, the relatively small amounts of virus factor released would bring about increased respiration. With increased respiration, which may indicate a generally increased cellular metabolism, viral growth rate would be accelerated. The larger amounts of influenza virus factor produced by the greatly increased numbers of virus particles would then depress oxygen consumption. Viral growth would then be retarded and remain at low rates until the "virus factor" was detoxified or otherwise rendered innocuous. The process would then be repeated. The low infectivity titres at 40°C may be a reflection either of destruction of protein and enzyme or a shift in the mode of metabolism or both.

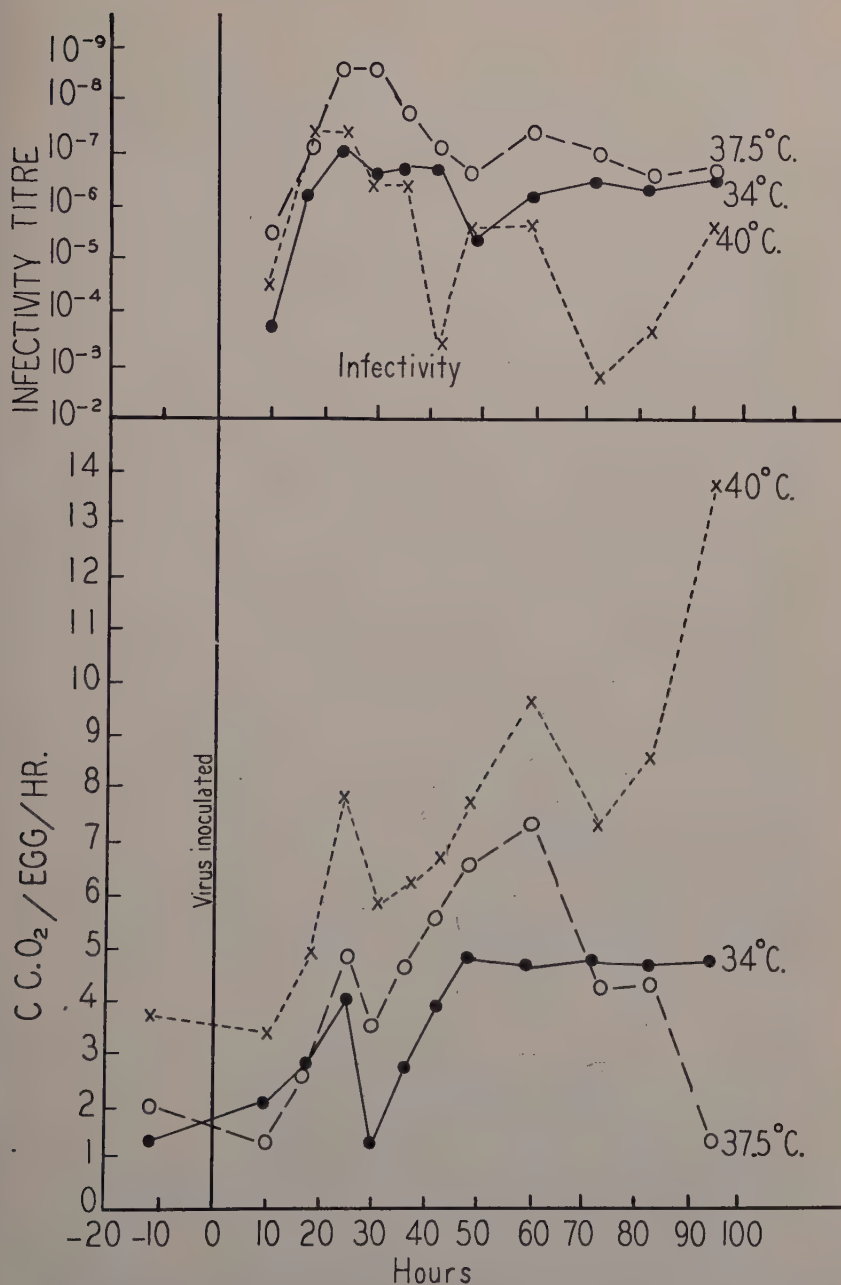


FIGURE 4. The effect of virus multiplication at different temperatures on the infectivity titer and oxygen consumption curves of embryonate eggs.

McLimans and his co-workers,^{29, 30} using a single egg technique, were unable to demonstrate any significant stimulation in the oxygen uptake of eggs infected with Newcastle disease virus, or Western equine encephalitis virus. However, if the points on our oxygen consumption curve at 37.5°C which correspond to the time intervals of the experimental measurements of McLimans at 37.5°C are joined, similar curves are obtained. This would indicate the desirability of continuously recording oxygen consumption which would undoubtedly resolve many of these seeming discrepancies.

Ackermann¹ recently reported on the oxygen consumption of isolated uninfected and virus infected chorioallantoic membranes. Membranes of both groups were observed to have the same oxygen uptake, although virus growth was observed on the inoculated membranes. He found oxygen uptake of the "normal" membranes was relatively constant over a 27-hour period. These findings are in contrast to our work using actively growing embryos; since growing tissues utilize increasing amounts of oxygen, Ackermann's data indicate that a study of surviving, rather than growing tissue, was made. The metabolic activities of cells under conditions of survival and growth could presumably lead to different results.

A new variable has recently been introduced into metabolic studies using embryonate eggs.¹⁶ Fertile eggs obtained commercially, which for many years have been entirely satisfactory for rickettsial growth, suddenly failed to support active growth of this organism. We have studied factors which might have been responsible for this change, and have obtained evidence that it has resulted from the addition to standard poultry feeds of a new source of animal protein factor, namely, the mash remaining from the manufacture of vitamin B₁₂, aureomycin, terramycin, streptomycin and penicillin. We have found that when these supplements are fed to laying hens, the eggs show reduced susceptibility by the end of the third week. The multiplication of the influenza virus in fertile eggs is not appreciably modified by feeding antibiotic supplement.

Previous studies have shown that penicillin increases oxygen consumption of the embryonate egg.²⁰ The above facts emphasize the need for controlling closely the diet of the laying hens, if metabolic studies of the developing chick embryo are to be significant.

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MODIFICATION OF VIRAL MULTIPLICATION IN THE CHICK EMBRYO

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The intimate details of viral multiplication persist in their obscurity. Numerous and extensive investigations concerning this profound and intricate biological process, however, permit certain interpretations and concepts pertaining to its nature. Much of this information has been derived from studies carried out with bacterial viruses.¹ It has been clearly demonstrated^{2, 3} that certain of the principles are also true in the case of animal viruses.

Viral multiplication appears to be a cyclic phenomenon¹⁻³ composed of step-wise events, several of which, at least, are well defined. To modify or alter this phenomenon by affecting the cycle at any susceptible locus is the problem to be discussed. If the manipulation is the proper one, the end-product of multiplication, new viral particles, theoretically may be limited or increased. It will be pointed out that both of these modifications have been accomplished.

The points at which the synthesis of virus may theoretically be attacked in order to modify this process are enumerated in TABLE 1. For purposes of convenience, inactivation of the viral particle *per se* has been included in this schema, although arbitrarily separated from those factors which limit host cell-virus association by combination with virus in a union which does not irrevocably alter the virus. It is clear that these loci, commencing with virus-host cell association, correspond in time and space with the steps of the multiplication cycle, namely: adsorption of viral particles to susceptible host cells, penetration of infecting particles into these cells, synthesis of new viral particles, and, finally, release of new particles from their benefactor cells.

That viral multiplication can be modified by interrupting certain of these steps of the cycle is exemplified in TABLE 2. No attempt has been made to review the literature completely. Moreover, the psittacosis-lymphogranuloma group of viruses, which react to chemotherapeutic agents in a different manner from other viruses, are now classified with the rickettsiae by the taxonomists⁴ and therefore are not considered. It is obvious that characterization of the mechanisms involved in each experimental model becomes increasingly difficult as the events are followed into the unknown of the susceptible cell. The effects upon proteins produced by such chemicals as tannic acid⁵ and ethylene oxide⁶ are well known, and their action as antiseptic agents in these instances is neither surprising nor mysterious. Similarly, prevention of viral infection by binding of virus by specific antibody or by the protein heat labile serum component demonstrable in sera of normal animals⁷ are phenomena whose mechanisms permit direct investigation. The receptor-destroying enzyme obtained from culture filtrates of *Vibrio cholera*⁸, RDE, has as a substrate certain mucopolysac-

charides contained in the so-called cell receptors with which influenza-mumps-Newcastle disease viruses combine. It has been demonstrated⁹ that alteration of these receptors by RDE can prevent infection of the chick embryo by members of this group of viruses. Infection of the chick embryo by influenza A virus has also been inhibited by the injection of large quantities of apple pectin,¹⁰ which is said to act as an analogue of the host cell receptors for virus. Certain acridine compounds reduce multiplication of various animal viruses¹¹⁻¹³ which multiply in the chick embryo. The site at which these acridines produce their effect on synthesis of influenza A, influenza B, and mumps is not entirely clear, but it has been suggested that

TABLE 1
THEORETICAL STEPS AT WHICH VIRAL MULTIPLICATION MAY BE MODIFIED

1. Virus
2. Virus—Host Cell Association
 - a. Virus
 - b. Host Cell Receptors
3. Penetration of Host Cell
4. Host Cell Metabolism
5. Release of New Viral Particles

TABLE 2
MODIFICATION OF VIRAL MULTIPLICATION BY ACTION OF VARIOUS AGENTS

<i>Site of action</i>	<i>Virus</i>	<i>Material</i>
Virus	IAV	Tannic acid
Virus	IAV, IBV, MV, NDV	Liquid ethylene oxide
Virus association	All viruses	Antibody
Virus association	IAV, IBV, MV, NDV, vaccinia	Labile serum component
Host "receptors"	IAV, IBV, MV, NDV	RDE
Host "receptors"	IAV	Apple pectin
?	IAV, IBV, MV	Acridines
?	IAV, IBV, MV	Cortisone
Intracellular	IAV, IBV, MV, NDV	Virus interference
Intracellular	MV	Friedländer polysaccharide

these drugs modify viral multiplication by directly damaging the host cells which support synthesis of virus.¹²

That the multiplication cycle can be interrupted and the production of virus decreased, therefore, is indicated by the above examples. The opposite effect has likewise been accomplished. Cortisone is able to increase the yield of virus from chick embryos infected with influenza A or B, or mumps viruses.¹⁴ The mechanism by which this is achieved is not known but it appears possible that the effect is on the host cells, either to increase the efficiency of each cell for the synthesis of virus, or to render more host cells susceptible to infection.

Viral multiplication may be further modified so that the process is limited either by another virus¹⁵ or by a complex carbohydrate, the type-specific capsular polysaccharides of Friedländer bacilli.¹⁶ Multiplication in each

instance appears to be reduced by an intracellular effect on susceptible host cells.

That a virus, active or inactive, may interfere with the multiplication of another agent, although unrelated, has been described for numerous pairs.¹⁵ However, the fact that this phenomenon does not hold for any two viruses has been equally well documented.¹⁵ Although the mechanisms involved are complex and difficult to elucidate, certain important factors appear clear: (1) quantitative relationships between interfering and challenging viruses must be carefully considered and controlled^{15, 17}; (2) sufficient time must elapse between inoculation of the initial virus and the challenging agent to permit infection, or at least parasitism, of a sufficient number of susceptible cells^{15, 17}; (3) the interfering virus does not alter the capacity of the host cells to adsorb the second virus¹⁸; (4) penetration of susceptible host cells by the challenging virus is not prevented by the initial virus inoculated¹⁹; (5) the second agent inoculated may, under proper conditions, interfere with multiplication of the initial viral inoculum;¹⁷ in fact, inactive homologous virus employed as the second inoculum during the latent period may inhibit the first cycle of multiplication of influenza A or B viruses¹⁹. In effect, therefore, the interfering virus is usually a prophylactic agent but, under special circumstances, may limit a single cycle of multiplication once it has been initiated. These facts suggest the hypothesis that the interfering virus reacts with susceptible host cells to combine with, compete for, or utilize some host cell material or metabolic system which is also required for synthesis of the challenging agent.^{15, 18}

A similar type of phenomenon has been demonstrated with the aid of a complex bacterial polysaccharide.¹⁶ These investigations demonstrate that type-specific capsular polysaccharides of Friedländer bacilli inhibit multiplication of mumps virus in the allantoic sac of the chick embryo and also decrease the susceptibility of this host to infection. Experimental data indicate that the susceptibility of the chick embryo to infection with mumps virus may be reduced approximately a thousand-fold by the type B polysaccharide, termed FrB. Not only was a greater quantity of virus required to infect the embryo, but also the carbohydrate reduced the actual amount of virus synthesized. It was demonstrated further that the quantity of infectious virus in the chorio-allantoic membrane, as well as in the allantoic fluid, was decreased in proportion to the reduction of the hemagglutinin concentration. Results of experiments in which incubation of embryos was carried out for six days indicated that multiplication of mumps virus was inhibited when 10 to 10^4 embryo infectious doses of virus was inoculated, but not if 10^5 E.I.D. was employed. The mumps virus, however, which continued to multiply in subsequent cycles after the injection of FrB, was, in fact, a polysaccharide-resistant variant which could experience unrestricted multiplication in the presence of large quantities of FrB.²⁰ This variant was otherwise indistinguishable from the parent strain of virus except for a slightly decreased rate of multiplication.²⁰

The capsular polysaccharides of the Types A, B and C Friedländer bacilli are equally effective in the limitation of multiplication of mumps virus.¹⁶

Figure 1 is a line graph showing the relationship between Fr. B concentration (micrograms per embryo) and Hemagglutination titer (difference from controls, log). The x-axis is logarithmic, ranging from 1000 to 5 micrograms. The y-axis ranges from -3.0 to 0.0. Data points are shown as open circles (o) and closed circles (•). A line with 'x' markers represents the geometric mean. The geometric mean curve shows a peak at 10 micrograms per embryo.

Fr. B (micrograms per embryo)	Hemagglutination titer (log difference from controls)	Type
1000	-2.2	•
1000	-2.1	•
1000	-2.0	•
1000	-1.7	•
1000	-1.2	o
500	-2.4	•
500	-2.2	•
500	-2.1	•
500	-2.0	•
500	-1.7	•
200	-2.1	•
200	-1.7	•
200	-1.6	•
200	-1.5	•
200	-1.4	•
200	-1.2	•
200	-1.1	•
200	-0.9	•
200	-0.5	o
100	-1.9	•
100	-1.7	•
100	-1.6	•
100	-1.5	•
100	-0.5	o
100	-0.6	o
50	-2.4	•
50	-1.5	•
50	-1.1	•
50	-1.0	•
50	-0.9	•
50	-0.2	o
10	-1.3	•
10	-1.2	•
10	-1.1	•
10	-1.0	•
10	-0.3	o
5	-1.0	•
5	-0.9	•
5	-0.3	o

FIGURE 1. Effect of different quantities of capsular polysaccharide of Friedländer bacillus type B (Fr. B) on the hemagglutination titer of allantoic fluids obtained from chick embryos inoculated with 100 E.I.D. of mumps virus. Polysaccharide was injected intra-allantoically three hours after virus. Groups of four embryos were employed. Each end point represents the mean difference in titer between one experimental and two control groups. Results indicated by open circles were obtained in a single experiment and, because they deviate systematically from the other results, they were not included in the calculation of geometric mean. From data reported by Ginsberg, Goebel, and Horsfall.¹⁶ Reprinted with the permission of The Journal of Experimental Medicine.

alkali renders the product of hydrolysis without biologic activity with respect to mumps but still capable of reaction with specific antibody. Moreover, this alkali-treated polysaccharide can limit the effectiveness of native FrB when injected into the allantoic sac before the untreated carbohydrate.²¹

Relatively small amounts of FrB are necessary to inhibit viral multiplication even when injected three hours after virus.¹⁶ The results of experiments carried out to investigate this point are summarized graphically in FIGURE 1, in which the difference in the hemagglutination titers of the

polysaccharide-treated and control groups is plotted against the quantity of FrB injected three hours after 100 E.I.D. of virus. As little as 5 μ g of FrB per embryo inhibited multiplication to a significant degree. Although 1000 or 500 μ g per embryo reduced the titer of virus to a greater degree than 5 μ g it was not a proportionate relationship. Moreover, the degree of inhibition obtained with 200 μ g was not significantly greater than that observed with only 1/40th the amount of polysaccharide. It should be

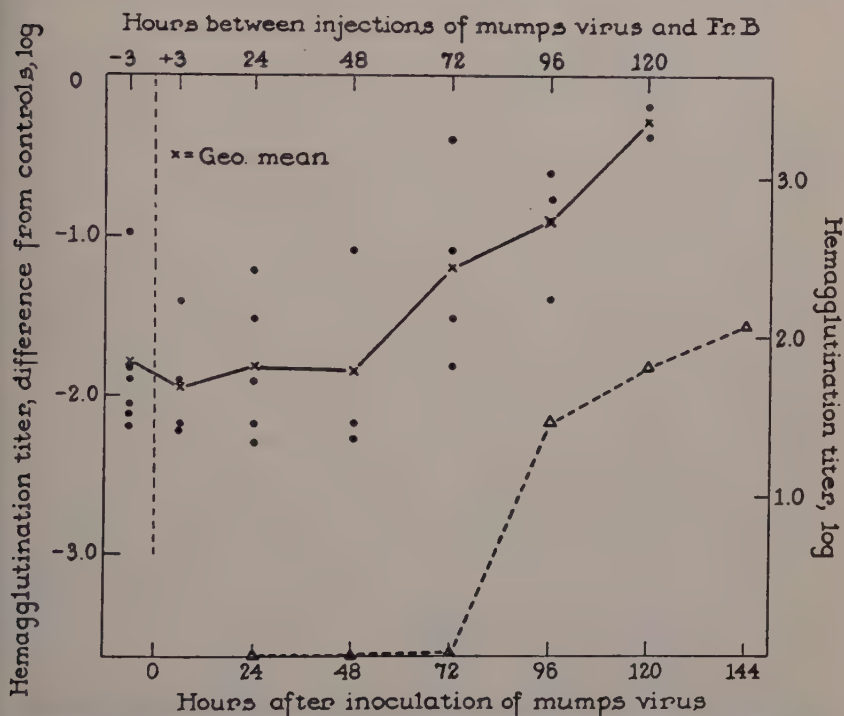


FIGURE 2. Upper graph: The effect of time between inoculation with mumps virus and injection of FrB polysaccharide on the hemagglutination titer of allantoic fluids obtained six days after viral infection. Groups of four embryos were employed; each end point represents the mean difference in titer between one experimental and two control groups.

Lower graph: The rate of increase in the hemagglutination titer of the allantoic fluid of control embryos inoculated with mumps virus. From data reported by Ginsberg, Goebel, and Horsfall.¹⁶ Reprinted with the permission of The Journal of Experimental Medicine.

emphasized at this point that although as little as 5 μ g of FrB per embryo reduced viral multiplication to a significant degree as much as 10 mg. of the substance had no demonstrable toxic effect on the host.¹⁶

Multiplication of mumps virus can also be inhibited after viral synthesis has commenced and infection has been well established.¹⁶ The results of experiments summarized in FIGURE 2 indicate that FrB injected three hours before virus was no more effective than when it was injected 48 hours after the onset of viral multiplication. Thereafter, the carbohydrate became decreasingly effective, but even four days after initiation of viral infection the final concentration of virus was significantly reduced. For the purpose

of comparison, there is shown in the lower portion of this figure the titer of mumps virus increasing with time in the allantoic sac when the embryo was inoculated with the same quantity of virus employed in the experiments described above, *i.e.*, 100 E.I.D. It will be noted that FrB was most effective when multiplication had not progressed to the extent that virus was demonstrable by the hemagglutination technique. It is important to emphasize that at no point did FrB reduce the concentration of virus already present at time of injection of the polysaccharide.

To inhibit viral multiplication after synthesis has commenced implies that a single cycle of multiplication can be interrupted. Direct evidence has been brought to bear on this point by the results of the experiment to be described. The characteristics of the multiplication cycle of mumps virus,²¹ as measured by the titer of soluble complement fixation antigen and infectious virus in the allantoic membrane, are similar to those for pneumonia virus of mice³: the latent period is of the order of 15 hours.²¹ Mumps virus, 10⁸ E.I.D., was inoculated into the allantoic sac of a large number of seven-day-old chick embryos and followed at intervals by 1.0 mg of FrB or saline into groups of 18 eggs each. Each group was subdivided into three groups of six, and allantoic fluids were harvested 24, 28 and 32 hours respectively after viral inoculation. Hemagglutinin titer of each fluid was determined. The results are summarized graphically in FIGURE 3. When polysaccharide was injected one or four hours after mumps virus, the yield of new particles from a single cycle was markedly reduced. The failure of FrB to produce significant effect when injected seven or ten hours after the latent period began suggests that this carbohydrate reacts with some host cell material or system utilized early in the multiplication cycle.

Examination of the steps of a viral multiplication cycle in relation to the inhibition of multiplication of mumps virus by Friedländer polysaccharides^{16, 21, 22} reveals that FrB does not react directly with the virus to inactivate it, does not combine with the virus, does not limit host cell-virus combination, does not prevent penetration of infecting viral particles, and does not restrict release of newly synthesized virus from host cells. The evidence which has been summarized led to the concept that the polysaccharide-inhibited multiplication by combination with, or competition for, some host cell material which is of prime importance for synthesis of mumps virus, is present in limited quantity, but is unessential for continued health of the cell.

FrB does not affect multiplication of influenza A, B, or Newcastle disease viruses, even when the polysaccharide is employed in relatively large quantities.¹⁶ This suggested the postulate that these agents multiplied by host cell pathways different from those traversed by mumps. If this were true, mumps should not interfere with the multiplication of influenza A, B, or NDV. Direct investigation revealed evidence to support this notion: mumps virus and influenza A or B multiplied concurrently in the allantoic sac of the chick embryo, even when mumps virus infection was initiated four days before inoculation of these latter agents.¹⁸ Conversely, the initial inoculation of influenza A or B did not preclude multiplication of mumps

virus. Furthermore, mumps virus interfered with multiplication of pneumonia virus of mice (PVM) in the mouse lung.²³ This agent is the only other known virus whose multiplication is inhibited by FrB.²⁴

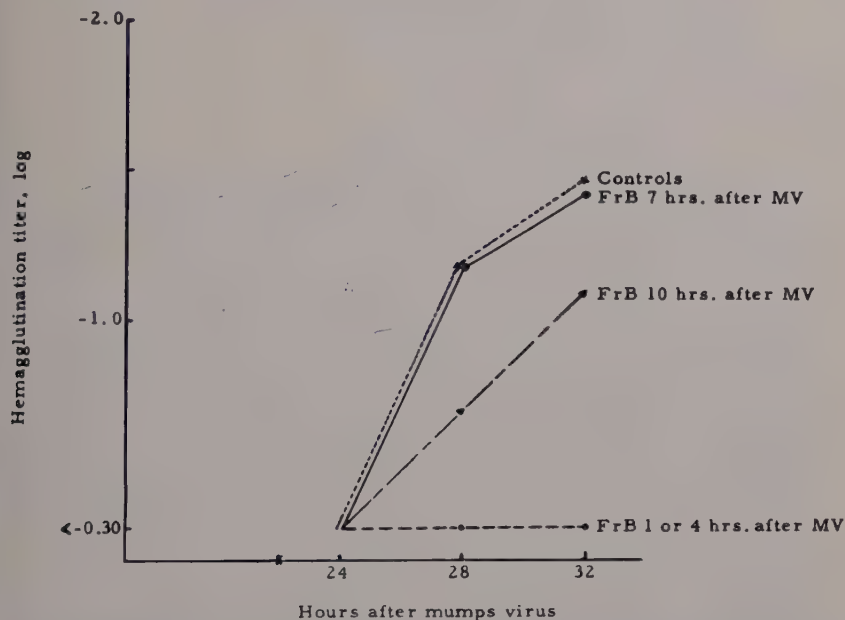


FIGURE 3. Effect of Fr. B polysaccharide upon the hemagglutination titer of allantoic fluid during a single multiplication cycle of mumps virus. Polysaccharide, 1 mg. per embryo, was injected intra-allantoically at different times after intra-allantoic inoculation of 10^8 E.I.D. of virus. Each end point represents the mean hemagglutination titer of allantoic fluids from six chick embryos.

TABLE 3
SUMMARY OF POLYSACCHARIDE INHIBITION VS. VIRAL INTERFERENCE

Virus	Polysaccharide inhibition	Successive infection			
		PVM	MV	IAV	IBV
PVM	+	-*	?†	+	+
MV	+	0	-	+	+
IAV	0	+	+	-	0
IBV	0	+	+	0	-

* Cannot be determined with active virus.

† Suitable host not available.

It appears now that viral interference and inhibition of viral multiplication by polysaccharide may be similar phenomena whose mechanisms, if not identical, are closely related. The former may be termed "biological interference" and the latter "chemical interference." This concept is summarized in TABLE 3. Multiplication of PVM and mumps is interrupted by FrB, and mumps interferes with PVM; the reciprocal cannot be tested.

Synthesis of influenza A and B viruses is not influenced by the carbohydrate, and although they interfere reciprocally with the multiplication of each other, they, in turn, can grow side by side with either PVM or mumps virus. Therefore, those viruses which react in like manner to FrB interfere with the multiplication of each other, whereas those which react differently to injection of this carbohydrate do not preclude the multiplication of the others.

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CHEMOTHERAPY TRIALS IN THE CHICK EMBRYO

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In this discussion of virus chemotherapy, I will not attempt to review all of the many leads and blind alleys that have been followed, nor am I able to announce the discovery of a new and all-curing antiviral. Rather, I will attempt to state in a simple way some of the problems involved and how the use of the developing chick embryo has aided the virologist in his continuing search for the philosophers' stone of a specific antiviral agent.

Since the advent of certain of the newer antibiotics, treatment of many of the bacterial diseases has developed primarily into a decision as to which of the several specific agents available should be tried first. Most of the virus infections, however, fail to respond to these newer drugs and relatively little progress has been made in their treatment to date.¹ It is true that benefit has been reported from use of this or that new wonder drug in diseases like measles, influenza, smallpox and certain others. On critical evaluation of these reports, however, it usually appears that the benefit resulted from control of secondary pyogenic infection and not from any suppressive action on the primary viral invasion.

It might be wise at this point to review some of the chemical and physical properties of viruses,² since an understanding of these is necessary to any planned chemotherapeutic approach.

First, and in regard to size, it is well known that the viruses are smaller than other infectious agents, but it is sometimes overlooked that the range of sizes within the group is quite extensive. As we pass from the smaller bacteria down the scale of size, we come to the *Rickettsia* and then to the largest viruses, the psittacosis group of *Myagawanella*. The latter, while true viruses in accordance with classical definition, show morphologic differentiation and are in the size range of 200–450 $m\mu$. Furthermore, like the rickettsiae, they have now yielded to chemotherapy with several of the newer antibiotics. Next in the scale, we have viruses primarily affecting the skin, such as variola, vaccinia, varicella, molluscum and all of the host of mammalian and avian "pox" viruses. This group ranges in size from 150–300 $m\mu$. and generally possesses a quadriform or brick-like shape with evidence of internal structure. In the next size range of 100–150 $m\mu$., we find herpes simplex and rabies. The next group is comprised of the elutable-hemagglutinating viruses, that include, among others, influenza and mumps. The members of this group have many properties in common and are about 100 $m\mu$. in size. Following in order are lymphocytic choriomeningitis and the encephalitides, yellow fever, dengue, foot and mouth diseases, and finally, the poliomyelitis group, these smallest viruses being not much larger than certain protein molecules. Thus we see a broad range of sizes with the largest representatives some fifteen to thirty-thousand times the mass or volume of the smallest, a range much larger than is encountered with the bacterial forms. With this range in mind it appears unlikely that any one antiviral substance will be found affecting the whole

group. Rather, we must look forward to a number of drugs, each useful in a small number of virus diseases.

Secondly, the viruses appear to have wide differences in biochemical structure. Many of the plant viruses and possibly some of the smaller animal viruses, such as rabbit papilloma, are relatively simple structures composed of protein and nucleic acid and well may be thought of as nucleoprotein macromolecules. With increase in size we find more complex structure, such as the presence of lipid in equine encephalomyelitis virus and of both lipid and free carbohydrate in influenza virus. Vaccinia and influenza appear to have a definite cell wall and even possess certain intrinsic enzymatic activity *in vitro*.

Thirdly, viruses will proliferate only intracellularly and cannot be grown on any inanimate, noncellular medium. They well may be described as the ultimate in parasites, being little more than reproductive templates borrowing all other aspects of life from their host cell. Little is known of this process whereby viruses reproduce within the cell. By analogy with bacteria, it might be assumed that they increase by binary fission, but this is probably not the case. Certainly the bacteriophages, or bacterial viruses, appear to break up upon entering a susceptible host cell and reproduce as components which later reassemble to form masses of the primary infectious particle.^{3, 4} These are then released to initiate a new cycle of infection. The weight of the evidence would appear to favor a similar mode of reproduction for many of the animal viruses.⁵⁻⁷ Whatever the mechanism, however, the fact remains that most of the process is contributed by the host cell and the infecting virus supplies only the final *modus operandi* to direct its own elaboration. While this minimal intrinsic activity is the viruses' greatest defense against successful chemotherapy, it may well turn out to be to our greatest advantage in the long run. When the key is found to the intrinsic viral metabolic process, as contrasted with the borrowed host metabolic pathways, then specific antiviral therapy will be available and the very simplicity of the virus particle that makes the search difficult will contribute to the effectiveness of the treatment agent.

Considering these factors we may now look for the weak spots in the chain of virus reproduction. The possibilities for interrupting the cycle are shown diagrammatically in FIGURE 1. In case A, the infective virus particle could be destroyed or inactivated before it reaches a susceptible cell. Viruses are sensitive to many of the common antiseptics, but these cannot be used in sufficient concentration, except under certain local conditions, because of toxic effects on the host. The classical treatment of the bite wound in rabies prophylaxis is an example. This approach is not likely to be profitable as we have seen that the infective virus particle is almost devoid of extracellular metabolic activity.

In case B, the virus particle is blockaded so that it cannot attach to the cell. This is probably the effect of neutralizing antibodies but the problem is to attain passively a high enough concentration of antibodies *in vivo* to block or neutralize all of the infective particles. Early in the infection, or prodromal period, when virus concentration is low, this is sometimes feasible,

as in the modification of measles by immune globulin or in the serum prophylaxis of rabies. The use of antiserum in treating established virus infections has been consistently disappointing, however. Another possibility, applicable in the case of viruses having demonstrable enzymatic activity in the free form, is to block these enzymes by supplying an excess of substrate, such as certain mucins⁸; or a great excess of cell-receptor analogue, such as apple pectin⁹, in the case of influenza or similar viruses. This approach, while workable in a limited space such as the allantoic cavity of fertile eggs, does not offer much promise because of the unattainable concentrations that would be required clinically. A more workable modifica-

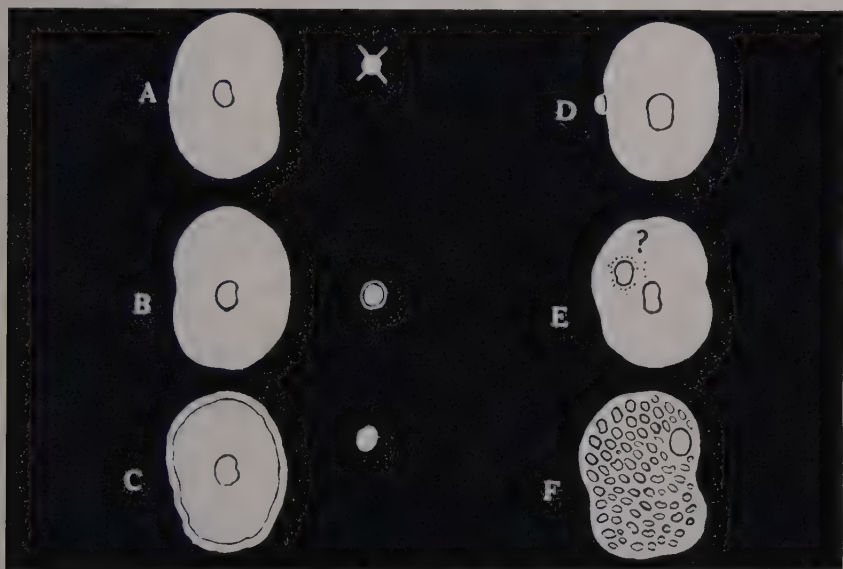


FIGURE 1

tion would be to supply the substrate in such a modified form that it could not be destroyed enzymatically. An approach to this possibility has been made experimentally in the use of periodate-modified mucins in inhibiting infection by influenza virus.⁸

The third and fourth possibilities (C and D) are to modify the susceptible cell so that the initial attachment of the virus to the cell could not occur, or, if it did occur, to block penetration into the cell. Such an effect can be demonstrated by the use of the receptor-destroying enzymes of certain bacterial species (RDE) to block infection with influenza virus in embryonated eggs by destroying the cell receptor points on the chorioallantoic membrane.¹¹

Another possibility is to utilize the mechanisms involved in so-called virus interference.¹² In this phenomenon, certain essential constituents of the cell are blocked to an infectious virus, either by inactive virus or by the

presence of another virus already infecting the cell. This effect is utilized in the treatment of distemper with distemperoid, or modified distemper virus,¹³ and it is possible that inactivated rabies virus may play a somewhat similar role in the Pasteurian prophylaxis of rabies. It is not unlikely that this approach may find further applications in prevention or treatment of virus diseases, but it implies finding some relatively harmless virus that would interfere with infection by the pathogen or the administration of very large amounts of inactive virus.

Where the virus has invaded the cell and is multiplying, we have other possibilities in addition to the two above. The first is to depress the metabolic activities of the host cell that are essential to viral multiplication so that the infection is stopped; or, second, to attack the vital contribution of the virus particle to its own reproduction. The former approach is much in vogue at present and the study of substances inhibiting specific cell enzyme systems is being undertaken in many laboratories with some indications of a certain degree of success. It seems unlikely, however, that the vital enzyme systems of invaded cells can be sufficiently modified to suppress virus growth without seriously affecting the activity of healthy cells. Is it possible that the cure will be worse than the disease?

The other possibility, finding means to block the specific viral factors involved in multiplication or reduplication and release of myriads of infectious particles, seems to offer the best chance of unqualified success. It is possible that the capsular polysaccharides of *Klebsiella pneumoniae*⁹ may exemplify substances of this type. To find such agents will be a difficult but not impossible task, as we have seen that the virus probably contributes little to its own reproduction, but must contribute some vital part. At present, we know little, if anything, of the biochemistry of virus multiplication, and we must look to the screening program for results therefore. Only by the systematic trial of large numbers of substances under controlled experimental conditions can we expect to uncover the vital key, and the key, when obtained, will itself furnish information on its mode of action.

It is here that the use of the developing chick embryo offers most promise, since the embryonated egg, to the virologist, stands in place of the bacteriologist's tube of culture medium. By the use of some of the techniques that have been described in the course of this conference it is possible to establish and maintain an extensive program to investigate the possible effect of many heterogenous substances on infections with certain representative viruses. For the remainder of my time I will outline such a program that is being maintained at present in our laboratory.

The first consideration is to maintain a screening program adequate to handle the anticipated supply of materials for test without overloading the facilities or compromising the essential confirmatory and evaluation activities. The primary screening tests themselves need not be elaborate or even statistically adequate since they are designed to answer only one question: Does this material have possibilities worthy of further study? They should cover more than one virus and the viruses chosen should be as widely representative of the whole group as possible. The tests should be self-controlling

and readily interpretable by some easily observable, objective criterion. The individual tests should be completed in less than seven days to allow regular weekly schedule and should give reasonably reproducible results over an extended period. One should be well aware of the calculated risk that potentially worthwhile compounds of low grade activity may be overlooked, but that this risk is compensated for by the greater number of materials that can be tested.

To meet these criteria one can develop a procedure utilizing four or more viruses. Vaccinia, eastern equine encephalomyelitis, the Lee strain of influenza, and meningopneumonitis are examples. These agents, while they are not viruses chosen on the basis of clinical importance, represent quite a broad size range and varied trophism. They can be adapted to produce a uniformly fatal infection in chick embryos following yolk sac administration, and with the proper infecting dose the time of embryonic death is quite uniform. Furthermore, all are very stable when stored in the frozen state and a large stock of standardized seed material can be prepared for use over a three- to four-month period.

The tests are all performed in the same manner. A large group of embryos of the proper developmental age are infected via the yolk sac with a predetermined dose of virus and returned to the incubator. Six hours later, the eggs are candled and arranged in groups of four for treatment. Each group of four eggs receives 0.5 ml. of a different material, also via the yolk sac, and at least sixteen eggs are treated with saline as controls. The embryos are then candled twice a day and the time of embryonic death recorded. The over-all mean death time of the whole group is calculated as well as that of the saline-treated control eggs and compared with the death time of the four eggs in each group treated with the test materials. Any delay of a half-day or more is considered worthy of retest.

It is debatable whether one should follow the usual practice and perform a preliminary toxicity test. The reason for this is obvious, since any toxicity test worthy of the name would be more elaborate than the chemotherapeutic trial itself. Unknown biological mixtures, such as crude antibiotic preparations, are tested in our laboratory, using 0.5 ml. of a Seitz filtrate. 0.5 mg./egg is used for the chemicals. These levels may be considered low but it should be remembered that the criterion for retesting is very lenient and retests are performed at both larger and smaller doses. When toxicity is encountered, the material is retested at lower concentrations until a satisfactory test is obtained.

By utilizing the test in embryonated eggs in this way we are able to survey a very large number of materials for potential antiviral activity and then evaluate the leads obtained in more elaborate experiments in eggs as well as animals. These primary screening tests in chick embryos, as indicated in FIGURE 2, are the keystone of our whole antiviral program. They do not supply the final answer because clinical effectiveness can only be determined by clinical trial, but they do indicate where to apply all the more elaborate experimental chemotherapeutic techniques that would be hopelessly cumbersome for indiscriminate use. Before leaving this subject,

I would like to comment on the secondary antiviral screening. This part of the program covers mumps, poliomyelitis, rabies, yellow fever and six other viruses. These tests are conducted in animals or in eggs in a more elaborate manner than that used in the primary screen. They are utilized on all materials showing any evidence of definite antiviral effect from the primary tests or on those that for theoretical reasons are considered worthy of more extensive evaluation. Following the routine secondary antiviral screening and further confirmatory studies in animals, pharmacological and toxicological studies are made and the biologic activity further defined prior to clinical trial. Thus in the over-all blueprint for chemotherapeutic studies

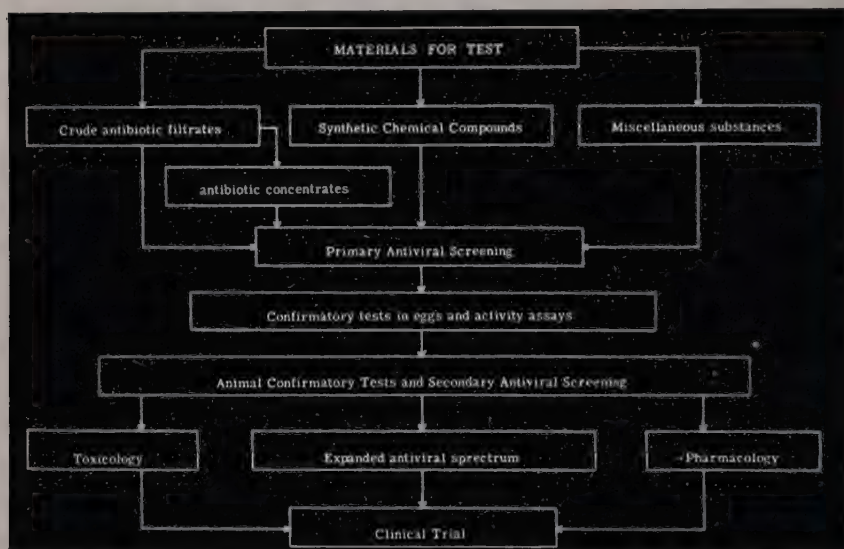


FIGURE 2

of potential anti-viral compounds we see that the use of embryonated eggs play a vital role. Without them the whole program would bog down for lack of a preliminary evaluating procedure whereby indications of specific viral inhibiting potential could be determined.

The program as outlined is still inadequate. I would like to see a representative of the smallest viruses in the primary screening but so far none is available that meets the requirements. There are probably a number of changes and improvements we could make in the technique of the tests to make them more sensitive. The use of tissue culture possibly will supplement the egg to broaden the scope of our initial investigations. Utilizing the chick embryo, however, we have now the techniques for a practical and extensive screening program that someday, in the not-too-distant future, with a reasonable degree of luck, will supply us with new chemotherapeutic agents for many of the infectious diseases still resistant to our present drugs.

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BACTERIAL AND MYCOTIC INFECTIONS OF THE CHICK EMBRYO

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The chick embryo is susceptible to infection with many pathogenic bacteria and fungi. This fact was quickly recognized by Goodpasture as a result of the early experiments which established the embryo method for the study of virus infections.^{1, 2} Consequently numerous investigations on bacterial infections of the embryo were undertaken concurrently with those which were devoted to virus infections. It was soon apparent that inoculation with various pathogenic bacteria caused disease in embryos. The different disease processes could be studied and compared at will during their various stages.

The first comparative study of this nature was reported by Goodpasture and Anderson.³ The chorio-allantois of 12- to 14-day-old embryos was inoculated with *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Streptococcus viridans*, *Eberthella typhosa*, *Aerobacter aerogenes*, *Corynebacterium diphtheriae* and *Brucella abortus*, respectively. Each microorganism induced a characteristic membranal lesion which in several respects resembled that which is observed in the human disease. Of particular note was the observation regarding the behavior of *E. typhosa* in the membrane. It was found to be capable of invading the cytoplasm of the living entodermal epithelial cells of the membrane, multiplying intracellularly, and forming small intracytoplasmic colonies. A subsequent study of the early intestinal lesions of human typhoid fever demonstrated that intracellular parasitism comprised an important phase of the early stages in the pathogenesis of typhoid fever.⁴ The young plasma cells in Peyer's patches and the lymphoid follicles of the ileum were found to be an essential host for the bacillus, serving as a nourishing and protective medium during the incubation period and throughout the active course of the disease.

The studies on the generalization of vaccinal infection following membranal inoculation directed attention beyond the chorio-allantois to the embryo.⁵ The development of lesions in various tissues and organs emphasized an additional approach to the study of the infectious process. The discovery of various routes of inoculation providing utilization of different portals of entry to the embryo broadened the experimental approach to the problem.⁶ Inoculation of the amniotic sac was proved to be particularly advantageous because the respiratory and alimentary tracts are accessible as portals of entry for different microorganisms by this means.

Interesting and important observations were made by Gallavan on *H. influenzae* and by Gallavan and Goodpasture on *H. pertussis* infection of the embryo.^{7, 8} Inoculation of the 11- or 12-day chorio-allantois with *H. influenzae* produced a relatively mild acute inflammatory membranal reaction in response to the local proliferation of the bacteria. In a significant number of these embryos, invasion, by way of the blood stream to the

meninges and cerebrum, took place. The development of the meningitis ependimitis and focal cerebral hemorrhages, which could be followed over a period of five days, was in most respects a counterpart of that observed in children. The intracranial lesions were found to begin as focal capillary hemorrhages which constituted points of entry for the microorganisms into the meninges and cerebrum. The bacilli proliferate rapidly at these sites, inducing extensive areas of cerebral hemorrhage and necrosis and a generalized acute inflammatory reaction in the meninges.

H. pertussis was observed to multiply readily in the chorio-allantois producing a local area of hemorrhage, necrosis and acute inflammatory reaction. Presumably as a result of direct extension of the infection to the amniotic fluid, the mouth and nasopharynx served as portals of entry to the trachea and bronchi. The same effect could be achieved by direct intra-amniotic inoculation. Significantly, extensive proliferation of *H. pertussis* was not observed in the respiratory tract until the 15th day of incubation. At this phase, ciliated columnar epithelium first lines the trachea and bronchi and is found temporarily in the esophagus. The embryo develops specific lesions only when this specific type of cellular differentiation is achieved. The environmental conditions provided by the cilia specifically support the growth of *H. pertussis*. The toxins elaborated by the bacilli exert their characteristic effect, producing midzonal necrosis of the tracheal and bronchial epithelial lining and stimulate a moderate acute inflammatory response. Occasionally a frank pneumonitis develops. These striking reproductions of the essential lesions of the human disease emphasize the existence of the highly specialized host-parasite factors which underlie the pathogenesis of whooping cough. The remarkable experimental reproduction of the essential lesion of the disease with pure culture substantiated the contentions regarding the specific etiological role of *H. pertussis*.

Embryos are susceptible to infection with the meningococci and gonococci. These closely related microorganisms induce lesions in the embryo which are indicative of differences in their behavior which correspond closely to their pathogenicity for the human host. Buddingh and Polk^{9, 10} studied meningococcal infection which developed following inoculation by the membranal, intra-amniotic, subcutaneous, intracerebral and intravenous routes into 12- and 15-day-old embryos. The younger embryos usually succumbed to infection within three to four days. A disease comparable to fulminating meningococcemia develops. No marked predilection for the meninges is observed. Vascular injury is produced because the meningococci appear to adhere to endothelial cells at numerous points. Petechial hemorrhages and extensive extravasations result either from injury produced directly by the bacteria or from the formation of bacterial thrombi. Fifteen-day-old embryos are much more likely to develop only a purulent meningitis regardless of the route of inoculation followed. Inoculation into the amnion especially provided an opportunity for tracing the pathogenesis of the meningitis. Under these circumstances the nasopharynx constitutes the portal of entry. Practically every embryo develops a pharyngitis and para-

nasal sinusitis. Invasion of the meninges occurs in a significant number. Careful study indicated that the meningococci gained entry to the meninges by way of the blood stream. There was no evidence of a direct transcranial extension from the infected sinuses to the meninges. Direct extension from the nasopharynx to the lungs frequently produces a bronchitis and pneumonitis.

Bang¹¹ found that gonococcal infection of the chorio-allantois induces a superficial lesion without much tendency for generalization to the embryo. Inoculation by way of the amnion incites an acute inflammation of the paranasal sinuses, which may extend to the embryonic lung and also involve the surface of the lining of the air sacs. Hemorrhagic reactions do not develop. Serial passage in the chorio-allantois sufficiently modified one strain of gonococci so that it killed a large percentage of 10- to 14-day embryos. In one of these embryos, a bacteremia and meningitis was demonstrated.

A comparison of infections of the embryo with *P. tularensis*, *P. pestis* and *Brucella abortus*, *suis* and *melitensis* reported by Buddingh and Womack revealed interesting basic differences in behavior of these bacteria.¹² These observations were made in embryos of 12-day incubation inoculated on the chorio-allantois. The embryonic infection especially emphasized the behavior of these microorganisms in relationship to the host cell. In this respect they exhibited varying degrees of facultative intracellular parasitism.

P. tularensis is highly pathogenic for the embryo. Following membranal infection none survives longer than 80 hours. During the first 24 hours, the infection remains localized in the chorio-allantois. There is evidence of limited extracellular proliferation of the bacilli in small areas of membranal injury or collections of extravasated red blood cells. In this initial period, however, invasion of ectodermal epithelium occurs. The bacteria grow within the cytoplasm of these cells where they multiply to great numbers. This feature is typical of the behavior of *P. tularensis* in the hepatic cells and endothelium of guinea pigs and within the epithelial lining cells of the wood tick. Following the primary intracellular multiplication dissemination by way of the blood stream takes place. Focal areas of necrosis develop especially in the myocardium, liver, spleen and kidneys. In the majority of these lesions, the microorganisms appear to grow extracellularly although occasional hepatic and glomerular endothelial cells contain intracytoplasmic bacilli.

The plague bacillus which also is highly lethal for the embryo exhibits no faculty for growing within an intracellular environment. It multiplies with extreme rapidity in the intercellular fluids and readily invades the circulation. It localizes especially in the liver. The focal lesions which develop within the chorio-allantois and liver consist of clumps of bacilli about which a mild inflammatory reaction develops. Marked injury to blood vessels with profuse hemorrhages characterize the infection with *P. pestis*.

Marked contrasts were observed following infection of embryos with *Brucella suis*, *abortus* and *melitensis*. These microorganisms also behave as facultative intracellular parasites. *B. suis* exhibits a predilection for ecto-

dermal epithelium invading mesodermal fibroblasts and vascular endothelium; *B. melitensis* restricts its intracellular invasion to ectodermal epithelium. Invasion of the embryo can be observed with *B. suis* and *Br. abortus*. It occurs predominantly as an intracellular extension of infection along the endothelium of the large vessels leading from the membrane to the visceral channels. Marked involvement of the liver, myocardium, spleen and kidneys takes place. The liver and kidney lesions are characterized by the parasitization of the endothelial cells of liver and kidney and the hepatic parenchymal cells. *B. melitensis* grows most abundantly extracellularly on the surface of the chorio-allantois. The bacilli can be isolated by culture from the blood after the 24-hour period but no focal lesions in the embryo have been observed.

These studies were made with strains recently isolated from the natural host. Adaptation to the embryo by continued passage could conceivably have enhanced the virulence of these strains for the embryo, markedly shortening the period of survival after inoculation with each from 96-120 hours to 24-48 hours. Accompanying the adaptation, the differences in behavior of each type might become less apparent. It must be emphasized that these observations were made with only one strain of each type.

The studies of Cromartie¹³ on the infection with *C. diphtheriae* of normal and passively immunized chick embryos are of particular interest. The diphtheria bacillus multiplies rapidly when introduced into the amniotic fluid of 12- to 15-day embryos. It thus gains entry to the nasal cavity, paranasal sinuses, pharynx, trachea and lungs. In normal embryos, a mucous membrane lesion develops which in many respects is similar to that in human diphtheria. The bacilli grow in the fluid which bathes the mucous membranes in these areas. Extensive necrosis of the epithelial lining results. A mild inflammatory reaction develops beneath the ulcerated mucosa, but no phagocytosis of the microorganism is observed. Embryos inoculated in the same manner after passive protection with diphtheria antitoxin reveal a similar extension of the bacilli to these vulnerable areas. Necrosis of epithelial cells does not occur, however, and the bacilli are rapidly phagocytized by numerous polymorphonuclears which invade the area. In the passively protected embryos, the effect of diphtheria toxin is neutralized. Cellular injury does not result; the phagocytes are not destroyed or repelled; and the invading parasite is eliminated. These observations emphasized the primary role of the exotoxin in the pathogenesis of diphtheria.

An unusual example of a highly specialized host-parasite relationship was demonstrated with the infection of embryos with *Streptobacillus moniliformis*.¹⁴ The human disease develops as the result of the introduction of this microorganism by means of the bite of a rat. One of its chief clinical features is the occurrence of a migrating polyarthritis. Following inoculation of the chorio-allantois of 12- or 14-day-old embryos, the microorganism first multiplies locally. It soon gains entry to the blood stream from where it becomes localized in various joints of the embryo, inducing a characteristic purulent arthritis. In the embryo, the microorganism exhibits a facultative intracellular parasitism. The cells and the immediate environment of synovial lining appear to be most propitious for the growth requirements of

S. moniliformis. During the early stages of the infectious process, the microorganism is observed to be present predominantly within the cytoplasm of the synovial cells. The embryonic arthritis appears to be a self-limiting process. At 72 to 95 hours following inoculation, the microorganisms appear to be degenerating, and beginning regeneration of the destroyed synovial lining is observed.

Anderson, De Monbreun and Goodpasture¹⁵ demonstrated the value of the use of the embryonic yolk sac for the isolation and cultivation of *Donovania granulomatis*. Their studies provided strong support for establishing the etiological relationship between this microorganism and the disease *Granuloma inguinale*. Jiminez and Buddingh¹⁶ infected embryos with *Bartonella bacilliformis*. This microorganism was readily cultivated in the allantoic fluid of embryos incubated at 25–28°C. after inoculation. Snow and Anderson¹⁷ described infection of the chorio-allantois with *Hemophilus ducreyi*. Bang's studies¹⁸ on the synergistic action of *Hemophilus influenzae suis* and the swine influenza virus on the chick embryo were important in providing an experimental demonstration of the pathogenesis of swine influenza.

Infection of the chorio-allantois with various pathogenic and non-pathogenic fungi was first described by Moore.¹⁹ The inflammatory reactions produced in some instances were comparable to those of the natural disease. More recently, Brueck and Buddingh²⁰ have demonstrated that the yolk sac of seven- to eight-day embryos is especially suitable for the cultivation of several pathogenic fungi, including *Actinomyces bovis*, *Nocardia asteroides*, *Nocardia intracellularis*, *Blastomyces dermatitides*, *Sporotrichum schenkii*, *Coccidioides immitis* and *Histoplasma capsulatum*. Further studies on the pathology and pathogenesis of these infections are in progress. It has been found that this method is superior to artificial media for the isolation and identification of these pathogenic agents from cases of disease.

Discussion

The foregoing review again emphasizes the potentialities of the chick embryo for the study of the infectious process. Each of the investigations present only beginnings of what may be learned from these experimentally-induced diseases of the embryo. It is especially noteworthy that specific organs, tissues, fluids and specific sites of the developing embryo present each pathogenic microorganism with the nutritional and environmental conditions which are most favorable for its proliferation. Furthermore, the inflammatory response to the injury produced corresponds in most details with that occurring in the natural disease. These characteristic localizations provide opportunities for analysis of the specific nutritional, biochemical and biophysical factors which predispose localizations characteristic of individual infectious diseases. The sharp differences in effect of specific infectious agents at different stages of embryonic development challenge investigations into the problem of susceptibility to infectious disease in a uniform organism which is not endowed with the capacity to respond with the development of specific humoral antibodies.

It is not intended to discuss the various and valuable studies in which bacterial infections of the embryo have been used to evaluate the activity of chemotherapeutic agents. This approach deserves a more precise and minute analysis. Careful histological studies should provide more information regarding the specific action of these agents.

Neither systematically organized studies of bacterial and mycotic infections of the embryo nor such studies of viral infections of the embryo, under uniform conditions of dosage, experimental technique and at various stages of embryonic development, have been undertaken as yet. Enticing opportunities await those who will approach the problem of the infectious process by this method.

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MEMBRANE GROWTH AND FUNCTION

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The extraembryonic membrane system of the chick consists of the yolk sac; the amnion and the chorion; the allantois, part of which fuses with the chorion to form the chorioallantois; and the albumen sac, an extension of the chorioallantois. They are temporary living appendages of the embryo and serve as functional organs and as intermediaries between the embryo and its total environment, which includes the non-living matter of the egg as well as the physical world beyond the limits of the eggshell.

Origin of Membranes

The membranes have a common origin from the extraembryonic portion of the blastoderm. The amnion and chorion are derived from the extraembryonic somatopleure, the yolk sac and allantois from the extraembryonic splanchnopleure.

Development and Growth of Membranes

The yolk sac is the earliest membrane to form, and can be recognized on the second or third day as essentially a continuation of the intestine. In its growth it eventually encloses the entire yolk. Its surface area increases very rapidly up to the sixth day,²³ decreases gradually from that time until the 11th day, and then diminishes at a more accelerated rate. The apparent shrinkage of the yolk sac is correlated, of course, with the decrease in the volume of its contents.^{52, 58}

The area vasculosa occupies only half of the yolk sac on the sixth day but continues to invade the area vitellina until it comprises the entire membrane by the end of the 14th day.²³ The area vasculosa, therefore, is still growing, particularly at its periphery, after the 11th day, when its surface area starts to decrease.

There is no direct relationship between the area of the yolk sac membrane and its weight, which increases at a rapid and almost constant rate until it attains a maximum of about four gm. on the 15th to 17th day of incubation.⁹ Thus weight is still increasing throughout the time that surface area is decreasing, a fact which is explained by the thickening and folding of the inner wall of the membrane, and also by inclusion of absorbed yolk substance within the yolk sac cells and blood vessels.

After attaining its maximum weight at the middle of the third week of incubation, the yolk sac membrane starts to undergo regression and becomes rapidly lighter. On the day before hatching, it has lost 50 per cent of its weight.⁹ It is then retracted into the embryo's body, together with its contents, which represent about one-third of the yolk's original weight. The yolk stalk persists as a part of the duodenum.³¹

The first indication of amnion formation is seen in the middle of the second day, when the chorioamniotic head fold arises. The reduplicated extraembryonic somatopleure subsequently grows over the embryo from all sides,

and, at the end of the third day, the folds finally converge and fuse above the posterior portion of the embryo's body. The outer and inner folds separate from each other everywhere except along the line of fusion, the outer fold becoming the chorion and the inner the amnion. The region of fusion, the sero-amniotic raphe or connection, develops a lumen on the 11th day, and this event has important consequences.

The growth of the amniotic membrane is slow at first. After the eighth day, the growth rate accelerates to such an extent that the membrane attains its maximum weight of about 350 mg. on the 12th day.⁵⁵ The period of most rapid weight increase coincides with the period of maximum accumulation of fluid within the amniotic sac; therefore, the amnion does not merely stretch to accommodate its liquid contents. Our observations show that the size of the amnion is noticeably smaller than normal if the egg is not turned frequently during incubation,⁴¹ larger than normal if the incubation temperature is too low.⁵⁷

The development of the allantois starts during the second day with the appearance of an evagination of yolk sac endoderm beneath the vestigial primitive streak. Through the work of Gruenwald²⁶ and Zwilling,⁷³ it has been made clear that the allantois is derived from the ventro-posterior wall of this outpocketing, which is located posterior to the tailbud and to the tail fold when the latter forms; the allantoic primordium, therefore, is distinct from that of the caudal intestine, which is anterior to the tailbud.

As a small sac, the allantois grows out on its stalk into the extraembryonic coelom. After the fifth day, when its outer wall starts to fuse with the chorion, it enters a period of accelerated growth and completes its development by the tenth or 12th day. In its advance from the large to the small end of the egg, the allantoic sac requires six days to surround the embryo and the amnion, with which its inner wall fuses in the region of the sero-amniotic connection; on the ninth day, it has covered the yolk sac and begins to enclose the albumen; and, before the end of the 12th day, its folds have met around the albumen, to form the albumen sac.

The allantoic membrane reaches its maximum weight of about two gm. on approximately the tenth day.⁹ The growth of the allantois, it has been shown, depends in part upon the stimulus of mechanical distension provided by the fluid present within the sac on and after the fourth day. When the Wolffian ducts and the mesonephric excretions are prevented from reaching the allantois, the sac fails to expand.⁵

Incubation of the egg with the sharp end uppermost causes the allantoic sac to be displaced to the small end of the egg, as Dareste discovered in 1863.¹⁵ If eggs are not turned, the growth of the allantois over the albumen is delayed and sometimes arrested before the albumen sac is completely formed.⁴²

The albumen sac appears to consist of an extension of the chorioallantois, rather than of the inner wall of the allantoic sac. The inner wall of the albumen sac contains ectodermal, villus-like projections which absorb albumen. Nevertheless, the participation of the albumen sac in the disappearance of albumen is small. In the 11-day chick embryo, the albumen sac

has developed a broad projection extending toward the sero-amniotic connection.⁵⁵ Rupture of the albumen sac at this site allows a large part of the albumen to seep into the lumen now present in the sero-amniotic connection, and thence into the amniotic cavity.

From the fourth to the tenth day, the membranes together (FIGURE 1) comprise between 40 and 50 per cent of the living matter in the egg, by weight.³⁷ After the tenth day, the embryo grows very rapidly, and the proportion of living matter formed by the membranes correspondingly decreases, becoming about ten per cent on the 18th day.

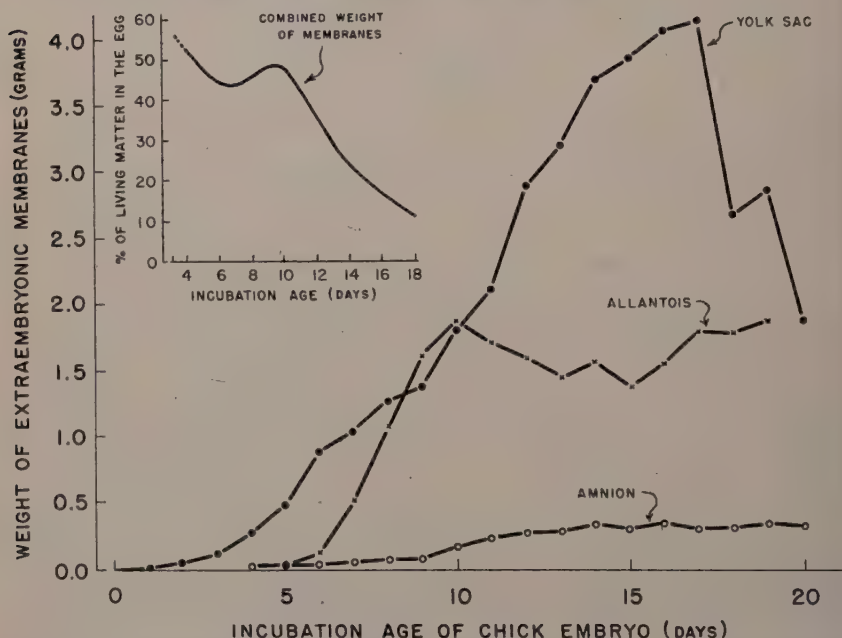


FIGURE 1. Weights of extra-embryonic membranes of the developing chick. (Yolk sac and allantois;⁹ amnion.⁵⁵) Insert chart shows the combined weight of the membranes as percentage weight of the egg's living matter.³⁷

There is a fairly close relationship between the growth and the metabolism of the membranes. A high rate of oxygen consumption (about 320 cu.mm./gm./hr.) is coincident with the most rapid increase in weight; thereafter, the respiratory rate of the membranes diminishes.³⁷ It is of interest to note that the percentage participation of the extraembryonic membranes in the respiration of the intact egg has been calculated and found to decrease in a sigmoid curve from 32 per cent on the fourth day to 4 per cent on the nineteenth.

All the membranes have the ability to continue their growth after the death of the embryo.^{8, 22, 48, 49, 64, 72} The amnion and allantois can survive for 11 to 15 hours at 45.5°C., a temperature that may kill the embryo in one to three hours.⁶⁴ The amnion has been known to live for eight to ten days after the early death of the embryo.^{72, 49} It continues to differentiate

and contract normally in the absence of blood circulation, although its inner, epithelial layer rapidly degenerates.

Functional Activities of Membranes

Yolk Sac. The chief functions of the yolk sac are the formation of blood, the absorption of nutritive material from the enclosed yolk, and the transport of the absorbed nutrients to the embryo. The last function is dependent upon the presence of a complex yolk sac circulatory system, whose development is closely related to blood formation.

Both blood cells and blood vessels arise from hemangioblasts,⁶⁰ which are aggregates of mesodermal tissue remaining after the separation of the exocoelomic walls from the so-called "blood islands." The cavitation of hemangioblasts begins at a very early stage and leads to the formation of spaces which coalesce into vessels containing blood plasma and the true blood islands. The latter are clumps of primitive blood cells said to be proliferated by the vascular endothelium;⁶¹ they give rise to erythroblasts on the second day. White blood cells, whose origin is disputed, begin to appear on the third day.

The capillary network of the yolk sac is established during a period of self-differentiation, before circulation begins. When blood starts to flow in the area vasculosa, at the 16-somite stage, the period of functional differentiation is initiated,²⁸ and the main arteries and veins form. The larger arteries develop from the portions of the network through which the blood passes most rapidly. It has been shown that the stimulus of blood circulation is necessary for the formation of the yolk sac arteries, since they do not develop in the absence of the heart.¹¹

When the yolk sac is deprived of blood circulation, the more central vessels undergo regression, although new vessels continue to form at the periphery of the area vasculosa.¹¹ At the end of incubation, the yolk sac arteries do not degenerate but die because of the contraction of the umbilical vessels.¹²

The vascular layer comprises the greatest part of the thickness of the yolk sac membrane. The blood vessels protrude into the endoderm of the wall, which is thus thrust into the mass of yolk underlying it. The radiating folds and ridges produced in this manner become especially pronounced after the ninth day of incubation and constitute an excellent absorptive surface.

The endodermal cells of the yolk sac secrete enzymes into the yolk; the cells then absorb the liquefied material and continue its digestion. Yolk is an emulsion of fat drops dispersed in a fluid, colloidal phosphoprotein,³² and, at the beginning of incubation, it is arranged in the form of spheres, each surrounded by a semi-permeable membrane. These spheres, when absorbed by the endodermal cells, push the nucleus to one side and surpass the cell protoplasm in volume.¹⁶ According to Grodzinski,²⁴ lipases dissolve the membrane of the yolk spheres and transform fats from glycerides to phosphatides; and proteases act upon the continuous phase of the yolk emulsion. Proteolytic activity is low in the yolk sac at the beginning, but it increases regularly and rapidly from the third to the ninth or tenth day and does not decrease thereafter.^{10, 45} Lipolytic activity is low until the fifth day, be-

comes intensified on the tenth day and attains a maximum on approximately the 14th or 15th day.^{44, 45} From this time on, the fat (ether extract) content of the yolk drops from 6.0 gm. to about 2.0 gm.⁵¹

Zwilling⁷⁴ has shown that the yolk sac performs the function of glycogen storage during the first week or ten days of incubation, and has thus corroborated Bernard,³ who detected glycogen in the yolk sac cells by histochemical methods.

Needham³⁸ has calculated that the yolk sac absorbs yolk at a very high rate during the first five days of incubation, taking up more than three times its own weight on the second day, for example. However, most of the material absorbed during the early period is utilized for the growth of the yolk sac itself. The rate of absorption (per unit of yolk sac weight) falls quickly after the fifth day and remains at a constant level. Starting on the sixth day, the amount of yolk taken up per day does not exceed the weight of the yolk sac membrane, which at this time requires but a small proportion of the absorbed material for its own metabolism.

The gross assimilation of yolk is shown by the changes in the weight and in the water and dry matter content of the yolk itself.^{53, 58} There is at first an actual increase in weight up to the tenth day, due to the diffusion of several grams of water from the albumen into the yolk. Thereafter water disappears rapidly. The dry material of the yolk undergoes a gradual but steady decrease, the rate of which accelerates considerably during the last few days. At the end of incubation, about five gm. of yolk remain. This is carried into the body of the embryo with the shrunken yolk sac, and most of it is absorbed during the first five days after hatching.

Amnion. The function of the amnion is predominantly to protect the embryo from injury, adhesions, and desiccation; this function is achieved through the presence of the amniotic fluid and through the muscular contraction of the amniotic wall, which is very thin. Also, the amniotic sac provides a channel for the assimilation of albumen by the embryo.

The amniotic muscles radiate from the umbilicus and extend two-thirds of the way to the mid-dorsal line,⁴⁰ where the cells grow in every direction in band-like groups⁶⁷ and where the resistance of the amnion to mechanical injury is greatest.⁴⁷ Contractions begin on the fourth day and increase in strength and frequency to a maximum on the 12th day (FIGURE 2); soon afterward, they abate and stop. Several types of amniotic contraction have been observed, but most frequently there is contraction and immediate relaxation of the radially arranged muscles, proceeding from one end of the amnion to the other and producing a generalized contraction wave.⁴⁰

The rate of amniotic contraction is affected by temperature,^{17, 25, 40} the most rapid and vigorous contractions occurring within the range of 38°C. to 42°C. Exposure to temperatures above 46°C. or below 25°C. usually causes contractility to disappear.

Fluid begins to appear in the amniotic cavity very shortly after the formation of the membrane, and, by the fifth day of incubation, it is present in sufficient amount to keep the sac tense.^{40, 46} The fluid continues to accumulate until it attains a maximum volume of three to six cc. or more on the

13th day or soon thereafter,^{30, 33, 57} when its weight is equal to eight or nine per cent of the egg's original weight.⁵⁷ Subsequently, the amount of amniotic fluid decreases very gradually until the 16th day, and then very rapidly.

The large volume of amniotic fluid normally present after the 13th day is explained partially by the entrance of the albumen sac contents into the amniotic cavity. The embryo then swallows the intermingled albumen and amniotic fluid, a fact which has been demonstrated experimentally. Such substances as lycopodium powder,⁶³ trypan blue,^{6, 27, 71} Evans' blue, charcoal, and radioactive chromic phosphate⁶⁶ are found in the intestinal tract

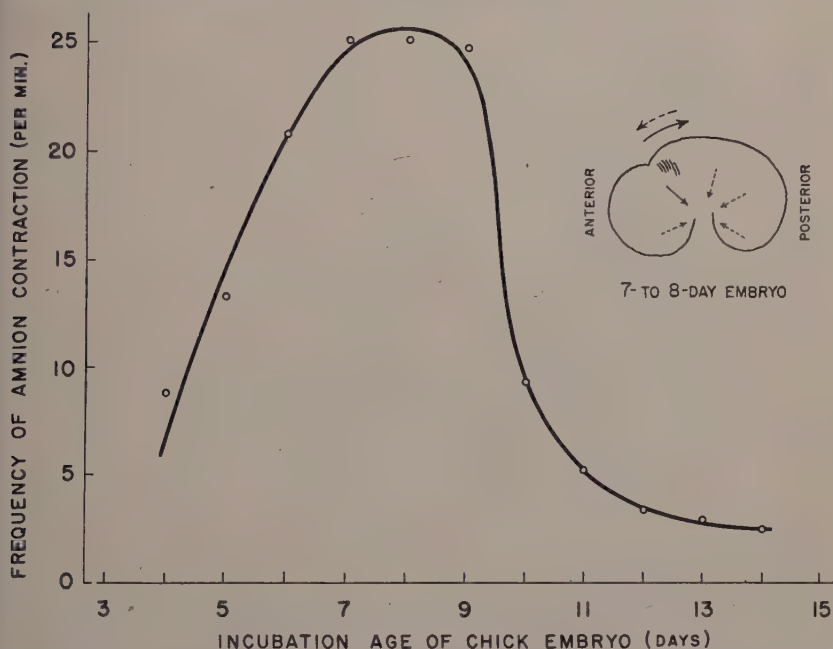


FIGURE 2. Frequency of amnion contraction during incubation of the chick embryo.^{33a} Insert diagram shows the direction and order of contraction of amniotic muscles arranged radially about the umbilicus.⁴⁰

of the embryo after being injected directly into the amniotic cavity. Trypan blue injected into the albumen at six and eight days has also been found subsequently in the embryo's digestive tract.²⁷

We observed that the amniotic fluid attains its maximum volume a day or two later than normally if the egg is not turned during incubation⁴³ or if the incubation temperature is too low (TABLE 1),⁵⁷ apparently because formation and rupture of the albumen sac is delayed under these conditions. In embryos incubated at elevated temperature (39.5°C.), there is an early peak in amniotic fluid volume on the ninth or tenth day, followed by a second and higher peak on the 15th or 16th day.⁵⁷

The influx of albumen into the amniotic sac is also demonstrable by the subsequent changes in the character of the amniotic fluid, which, in the early period, is very dilute, containing more inorganic than organic material; so-

dium and chloride predominate.³⁰ There is an increase in dry matter content,^{30, 75} and organic matter rises from less than one per cent to 29 per cent.³⁰ Total nitrogen, present in the amount of one mg. or less on the tenth day,

TABLE 1
INFLUENCE OF INCUBATING TEMPERATURE ON THE ACCUMULATION OF
AMNIOTIC FLUID AND ON ITS SPECIFIC GRAVITY⁵⁷

	Control at 37.5°C.	Experimental	
		at 34.5°C.	at 39.5°C.
Volume of amniotic fluid: appearance of peak	13 days	16 days	9 and 16 days
maximum value	3.8 cc.	3.9 cc.	2.4 cc. and 6.3 cc.
Specific gravity on 15th day	1.044	1.016	1.056

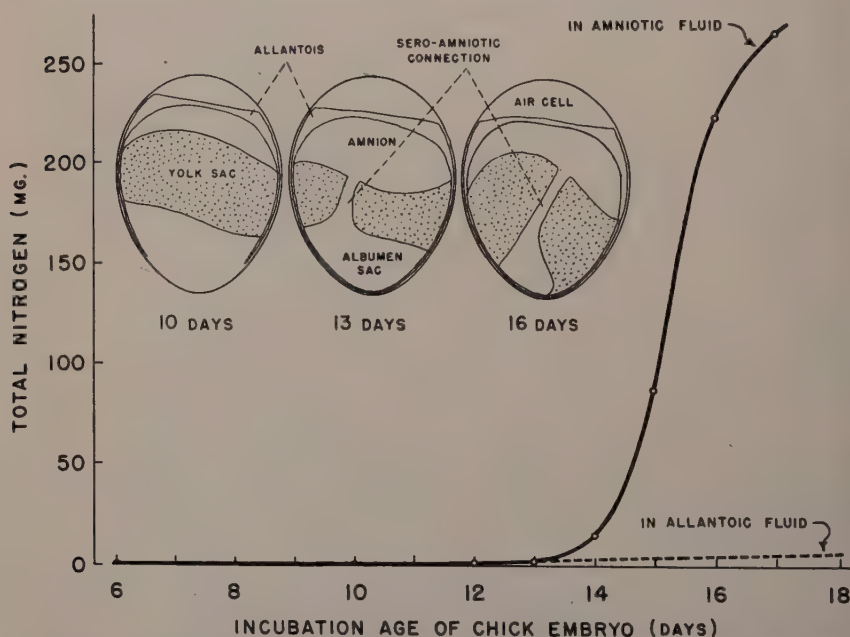


FIGURE 3. Total nitrogen content of amniotic and allantoic fluids of the developing chick.^{75, 65} Insert diagrams represent surface views of the contents of eggs which have been boiled⁵⁸ and from which the shell and allantoic membrane have been removed; three stages in the perforation of the albumen sac and the sero-amniotic connection are depicted.⁶⁵

may be found on the 16th to 18th day (FIGURE 3) in quantities varying from 100 mg.⁶⁵ to 250 mg.;⁷⁵ its amount per unit volume of fluid may undergo as much as a 600- to 800-fold increase.^{18, 75} The preponderance of total nitrogen is protein nitrogen,⁶⁵ and only a very small proportion is attributable to urea, ammonia,³⁰ and uric acid.¹⁸ Electrophoretic analyses of 14-day amniotic fluid indicate that the proteins contained in it are identical with those of egg albumen.³⁴

The specific gravity^{30, 42, 57, 75} and refractive index⁴² of the amniotic fluid reach maximum values on the 14th or 15th day of incubation, concomitantly with the gradual entry of albumen into the amniotic cavity. Subsequently, however, these values diminish, a fact which is somewhat difficult to explain but which, in combination with Fiske and Boyden's¹⁸ and Kamei's³⁰ data showing a decrease in concentration of total nitrogen on the 16th and 17th days, may indicate merely continuous replacement of the swallowed albuminous fluid by newly-secreted, more watery liquid.

Specific conductance also declines simultaneously with the entrance of albumen into the amniotic cavity.^{43, 69} This is probably due to decreased ion mobility or adsorption by protein molecules.

There is general agreement that the pH of the amniotic fluid undergoes a decline during the period when albumen is mingled with it and rises again late in the incubation period.^{2, 42, 57, 63, 70, 75} Usually the change in hydrogen-ion concentration is sufficient to cause the slightly alkaline fluid to become slightly acid in reaction before returning to alkalinity. The drop in pH is probably not a direct result of the influx of albumen, for albumen, although it becomes progressively less alkaline throughout incubation, tends to hover around the neutral point after the tenth day;^{7, 54} Aggazzotti,¹ however, reported the pH of albumen to be close to 6.0 on the 11th day. Walker⁷⁰ suggested that the drop in the pH of the amniotic fluid is due to the increased carbon dioxide production of the embryo and the diffusion of the gas from the embryo's tissues into the amniotic fluid.

It is of interest to note that the amniotic fluid possesses no buffer system on the eighth to 12th days, but, on the 14th day, buffering capacity is present.⁴³ This is due, no doubt, to the presence of albumen.

Allantois. The allantois is a respiratory organ and a repository for the excreta of the embryonic kidneys. The initial fusion of the chorion and the outer wall of the allantoic sac is followed immediately by the development of the vascular network which makes it possible for the chorioallantois to function in its respiratory capacity. Until the 11th day, this network lies immediately beneath the outer layer of the chorioallantois; but, as the result of prolific growth, it then forces its way through to the outer surface of the membrane, and on the 15th day, comes into direct contact with the inner surface of the shell membrane.¹⁴ The chorioallantois also contains a lymphatic network continuous with the lymphatic system of the embryo.

The capillary plexus fails to develop in the allantois if the latter is prevented from coming into contact with the chorion. Various incubation conditions also affect the position and extent of the chorioallantoic vascular pattern. If the egg is not turned during the developmental period, the vessels do not distribute themselves over the entire sub-shell area,⁴³ and, furthermore, they leave erosion grooves on the inner surface of the eggshell. These grooves, incidentally, are evidence that calcium is withdrawn from the shell for utilization by the embryo.²¹ Incubation under increased atmospheric pressure (40 lb.) retards the development of the entire allantoic circulatory system, so that 11 days are required to attain the eight-day stage.¹⁹ Remotti,⁵⁰ who subjected incubating eggs to an increase in partial

oxygen pressure to 50-70 per cent of total pressure for two to seven days, found that the density of the capillary network was reduced, although normal respiratory equilibrium seemed to have been maintained.

The allantoic sac begins to function in its excretory capacity as early as the fifth day. As Boyden⁶ showed, mesonephric excreta start to accumulate within it at this time, the fluid already present on the fourth day being presumably derived from the allantoic wall. Between the seventh and the 13th day, the volume of allantoic fluid increases from about 1.0 cc. until it attains its average maximum of approximately 6.5 cc., although any amount from 4.3 cc.⁷⁵ to 10.0 cc.⁴³ has been reported. Thereafter, there is an equally regular and rapid decrease in volume leading to virtual disappearance by the end of incubation.

Incubation at temperatures that are either too high or too low results in the accumulation of less than the normal amount of allantoic fluid (TABLE 2), the diminution in volume varying directly with the extent of deviation from the optimum incubation temperature of 37.5°C.^{57, 59} An increase in the volume of allantoic fluid has been observed after the introduction of influenza A virus into the allantoic sac.³⁹

Temperature also affects the time at which the allantoic fluid reaches its maximum amount, the volume peak being advanced or delayed as the temperature of incubation is elevated or depressed. At 39.5°C., the terminal decline in the volume of allantoic fluid may begin as early as the 11th day and coincides with an abnormal, late increase in the volume of amniotic fluid.⁵⁷

Chemical analyses of the allantoic fluid provide ample evidence that the allantoic sac serves as a receptacle for nitrogenous waste products. There is an 80-fold increase in total nitrogen between the fifth and the 13th day¹⁸ and a further tripling in amount between the 14th and the 18th day.⁶⁵ The largest single fraction of this nitrogen is due to the presence of uric acid, the chief excretory product of birds. Uric acid nitrogen represents a continually increasing proportion of total nitrogen up to the 16th day, at least,^{20, 65} and may constitute as much as 74 per cent by the 12th day,¹⁸ 90 per cent thereafter.³⁶

From the fifth to the 12th day, there is a precipitous rise, a 400-fold increase, in fact, in the actual amount of uric acid present in allantoic fluid (FIGURE 4), and, on the 19th day, as much as 100 mg. of uric acid may be present in the allantoic cavity.¹⁸ Because of the decrease in the volume of allantoic fluid after the 12th day and the low solubility of uric acid, precipitation of uric acid as urates takes place during the last week of incubation. At the end of incubation, nearly 90 per cent of the urates are found as solid deposits.¹⁸ Extremes of incubation temperature naturally increase the concentration of uric acid in the allantoic fluid, because of the diminution in volume.⁵⁹

Among the non-uric acid compounds in the allantoic fluid are creatine, in increasing amounts¹⁸ up to the 14th day;³⁰ amino acids, decreasing in concentration but increasing in absolute quantity;¹⁸ purine bases, diamino acids, and creatinine, which, according to Targonski,⁶⁵ increase from the 12th to

the 16th day and then decrease; and ammonia and urea, reaching maximum amounts on the 14th day.³⁰ Urea nitrogen is only about eight per cent of total nitrogen during the last two weeks,⁶⁵ ammonia even less; Needham³⁶

TABLE 2
INFLUENCE OF TEMPERATURE ON THE ACCUMULATION OF ALLANTOIC FLUID AND OF NONCRYSTALLINE URIC ACID⁶⁹

	Control at 37.5°C.	Experimental	
		at 34.5°C.	at 39.5°C.
Volume of allantoic fluid:			
appearance of peak	13 days	14 days	11 days
maximum value	6.2 cc.	1.5 cc.	2.0 cc.
Uric acid mg./100 cc. at the peak	90	140	135
Specific gravity ⁶⁷	1.005	1.004	1.028

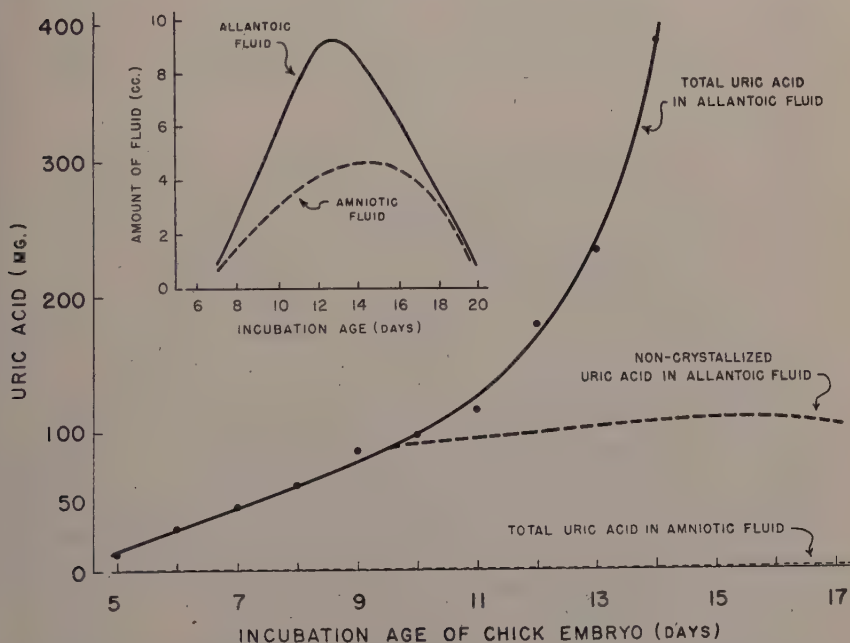


FIGURE 4. Uric acid content (total uric acid¹⁸ and non-crystallized uric acid⁶⁹) of allantoic and amniotic fluids. Insert chart shows total amounts of fluids.⁴⁰

has shown that, prior to the sixth day, urea is 60 per cent of total nitrogen excreted and ammonia 40 per cent, whereas uric acid is then only seven to 15 per cent, according to Fiske and Boyden.¹⁸

Various physical properties of the allantoic fluid reflect its changing constitution throughout incubation. The specific gravity and refractive index rise consistently throughout incubation, but particularly after the 14th day,

as would be expected in view of the increase in solid matter and the resorption of water. The pH declines progressively from a point close to pH 8.0 at the end of the first week or ten days^{42, 57, 63, 70} to the vicinity of pH 5 or 6 a day or two before hatching. The high original value is perhaps due to the relatively high level of urea,³⁵ acting as a weak base; the low final value is perhaps due to the combined effect of high uric acid excretion, increased carbon dioxide production, and, as shown by Kugler,³³ a rise in phosphate concentration. Infection with influenza virus tends to raise the pH of the fluid.³⁹

The increase in the concentration and absolute amount of phosphate (over 80 per cent of which is inorganic) in the allantoic fluid between the 11th and the 13th day³³ is perhaps related to the increase in specific conductance observed during the same period.^{56, 69} Specific conductance has been observed⁴³ to drop after the 14th day, a fact which cannot be attributed to dilution; it is probably caused by removal of ions from solution, either through the formation of undissociated salts of uric acid or through absorption. The data of Iseki²⁹ do, in fact, indicate that there is a decrease in potassium, sodium, calcium, magnesium, inorganic phosphate, silicon, and chloride between the 14th and the 18th day; Yamada's⁷⁶ data also show a decrease in chloride. A decline in osmotic pressure during this period^{4, 43} supports this explanation.

There is general agreement that the allantoic fluid is hypotonic to the amniotic fluid throughout incubation.^{4, 30, 43} According to Yamada,⁷⁶ this relationship is due chiefly to the presence of a higher concentration of chloride in the amniotic fluid, other minerals (potassium, calcium, magnesium, and inorganic phosphorus) being more concentrated in the allantoic fluid on the 13th day of incubation. On the other hand, Kamei's³⁰ data for the 14th day show exactly the opposite. The mineral composition of the allantoic fluid is altered somewhat after cortisone is injected into the yolk sac; sodium is increased and potassium decreased, an effect which is the reverse of that observed in man subsequent to the administration of cortisone.¹³

It is interesting to note that Yamada⁷⁶ attributes the movement of water through the developing egg to osmotic pressure differences due to varying concentrations of chloride in the yolk, albumen, allantois, amnion, and embryo. The migration of large amounts of water can be surmised from the fact that, according to Fiske and Boyden,¹⁸ 100 mg. of uric acid are excreted into the allantois in 20 days; from the solubility of uric acid, it can be calculated that to excrete this amount, about 1500 cc. of water would be required. Although it appears that water may diffuse out through the allantoic membrane, experimental evidence from our laboratory indicates that it does not pass from the amniotic sac into the allantoic sac. The injection of 0.5 ml. of molar phosphate buffer solution into the allantoic cavity on the eighth day produces an increase in the volume of allantoic fluid, due apparently to osmotic inflow. Very little change occurs, however, in either the volume or ionic concentration of the amniotic fluid, which therefore appears not to be the source of the additional water in the allantois.⁴³

This experiment, as well as those of Kugler³³ and Taylor and Saenz,⁶⁶

serves also as evidence that the allantoic membrane is impermeable to inorganic phosphate, since there was no increase in the phosphate concentration of the amniotic fluid despite a 90-fold increase in that of the allantoic fluid. There were no significant changes in pH, conductance or freezing-point depression of the amniotic fluid. It appears that the complex of extra-embryonic membranes can maintain physiological conditions even when the osmotic pressure of the allantoic fluid is abnormally high.

The permeability of the allantois to phosphate seems to be increased by influenza virus infection of the allantoic sac, which causes a decrease in the phosphate content of the allantoic fluid and an elevation in that of the embryo's blood. However, the permeability in only one direction appears to be changed, because the intravenous introduction of phosphate into the infected embryo does not produce an increase in phosphate in the allantoic fluid.⁶²

Summary

The foregoing discussion on growth and function of extraembryonic membranes of the chick leads to the following conclusions:

The membranes originate independently as highly specialized and structurally distinctive living appendages of the embryo. Growth and development proceed in accordance with the degree of participation in various functional activities. The chief functions of the yolk sac are the formation of blood, the absorption of nutritive material from the enclosed yolk, and the transport of the absorbed nutrients to the embryo. The functions of the amnion are to protect the embryo from injury, adhesions and desiccation, and to provide a channel through which albumen passes from the albumen sac into the digestive tract of the embryo. The allantois is a respiratory organ and a repository for the excreta of the embryonic kidneys. The chemical composition and physical properties of the amniotic and allantoic fluids give evidence of the roles played by the membranes containing them. The extraembryonic system of the chick is a dynamic, semi-autonomous organization of matter forming an inseparable part of a larger physiological system: the egg developing in isolation. Through the exercise of their individual properties of selective permeability to organic compounds and inorganic electrolytes, the membranes maintain the integrity of the several fluid and semi-fluid, chemically different phases of this system, and assist in preserving the harmonious interrelationships upon which ontogenetic processes depend.

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TRANSPLANTATION OF NORMAL EMBRYONIC TISSUES

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The method of transplantation has been extremely useful in the analysis of problems concerning the physiology of development of the chick. Through variations in its application and the perfection of suitable techniques for attacking particular problems, it has been possible to obtain information concerning causal relationships in developmental processes—information which has helped to a great extent to unravel some of the complexities of ontogenesis. The purpose of this paper is to review briefly the methods of isolation and transplantation that have been most frequently used for the study of the development of normal embryonic tissues and to present some of the principal findings which have come from their use.

Transplantation to the Chorio-allantoic Membrane. The technique of grafting isolated portions of embryos to the chorio-allantoic membrane has offered opportunity for an attack upon widely varied types of embryological problems and has been used more extensively than any other *in vivo* method. In many respects it presents ideal conditions for culturing tissues. The chorio-allantoic membrane of the chick is composed of mesenchyme bounded by an outer ectodermal, chorionic epithelium and an inner entodermal, allantoic epithelium. By the eighth day of incubation, it has become sufficiently vascularised to enable rapid incorporation of the graft. The nutritive requirements of the grafted tissues are ideally met, inasmuch as complete cytological and histological differentiation is permitted. Morphogenesis is suppressed to a great extent, probably as a result of mechanical conditions associated with limitations of space. Since the graft is not in actual contact with the body of the host embryo, the probability of inductive reactions is minimized. The graft, however, is connected with the circulation of the host and therefore is exposed to any hormones or other chemical agents that may be carried in the blood stream. The period of uninterrupted growth of the grafted tissue is fairly long, nine to ten days, or until the host begins to hatch.

The chorio-allantois was first brought into use by Rous and Murphy (1911) for studying the growth of abnormal tissues (rat and chick sarcomas). It was soon taken over almost entirely by embryologists for studying embryonic differentiation. Among the pioneers in this field were Dantchakoff (1916), Hoadley (1924), and Willier (1924), who used the method extensively for investigating problems centering around the developmental potencies or the differentiation capacity of areas of the early chick blastoderm.

Some results obtained by Willier (1926) from experimentation with chorio-allantoic grafts of whole blastoderms of the head-process stage suggested that Hensen's node played an important role in the formation of axial organs in normal development. This clue led to a systematic investigation, by Willier and his students, of the origin of axial and non-axial organs in stages ranging from the unincubated (pre-streak) blastoderm through the forma-

tion of somites. From the results of these investigations, particularly those of Hunt (1931), it was demonstrated that Hensen's node is a center of development and essential to the formation of the embryo. As analyses of the developmental potentialities of parts from various axial levels of the early blastoderm proceeded, it became increasingly evident that particular structures, such as brain, ear, eye, heart, liver, thyroid, mesonephros, gonad, *etc.*, develop only from rather definite regions or axial levels. Owing to the fact that even very small pieces of the early blastoderm, primitive streak, and head-process stages undergo normal histogenesis in the chorio-allantois, it became possible to divide one blastoderm into as many as 18 portions and to test the potencies of each measured piece separately in grafts. Data obtained by this method were used to construct a potency map showing the location of the various organ-specific areas (Clarke 1936; Rawles 1936; 1943; Willier and Rawles, 1935). Each organ-specific area occupies a definite spatial position in the flat blastoderm with respect to the anterior end of the streak and Hensen's node, and each exhibits the characteristics of an embryonic field. The localization of many of these areas corresponds in a general way to the prospective significance maps of Wetzel (1929) and Pasteels (1937), constructed from data obtained from the use of vital staining methods. (See Rudnick, 1944, for a comprehensive discussion of the various mapping methods.)

The method of transplanting tissues to the chorio-allantoic membrane has been used successfully, not only to elucidate problems of early embryonic organization of the chick blastoderm, but also to study the development of virtually all of its individual organs and organ systems. The general procedure has been to isolate a particular primordium at successive stages in development and to allow it to continue its development in the chorio-allantoic membrane. By so doing, the time of origin of specific organ-forming potencies may be ascertained. The gonad, for instance, has been quite thoroughly studied (Willier, 1933). Results have clearly shown that there is a progressive change in the organization of the primordium as it undergoes morphogenesis into a genital ridge. Once the genital ridge stage is attained, the gonad is specifically organized as to sex and, in the case of the female, as to laterality also. The important discovery that a gonad (testis-like) may differentiate entirely free of primordial germ cells was also made from a study of chorio-allantoic grafts (Willier, 1937).

The successful results obtained from grafting normal chick tissues led to an attack of similar problems of differentiation in the mammalian embryo. Since experimental studies on the early embryos of mammals are hindered by technical difficulties associated with intra-uterine development, grafting to the chorio-allantois seemed to be one way of overcoming these difficulties. Hiraiwa (1927) was the first to apply this method to the study of embryonic rat tissues. A much more thorough and extensive investigation was made later by Nicholas and Rudnick (1933). They tested the proliferation and differentiation capacity of various embryonic tissues in a closely graded series of stages ranging from six to eleven days. Normal histological differentiation was attained in many instances. Organogenesis and embryogene-

sis were suppressed to a great extent as in embryonic chick grafts. Tissues of the rabbit embryo behave on the chorio-allantois very much like those of the rat (Waterman, 1936). Human embryonic material has also been used with success (Dantchakoff and Gagerin, 1929). That successful transplantation can be made between embryos of widely unrelated individuals such as birds and mammals, indicates that there is no essential chemical incompatibility between their tissues and tissue fluids. From the investigations of Sandstrom (1934) on tissue specificity in chorio-allantoic grafts, there appears to be no sudden development of a general antagonistic reaction against heteroplastic transplants in the chick at any time before hatching. If any reaction does occur, it is so gradual that the tissue growth is unaffected. The fact, then, that embryonic tissues show so little, if any, species specificity has made them excellent material for all types of transplantation studies.

In vitro Cultures. Waddington (1932) introduced a method of cultivating whole blastoderms, or isolated portions, on the surface of blood plasma clots *in vitro* which has also proved useful in the investigation of many problems concerning embryonic differentiation in the chick. In this country it has been used rather extensively and with a great deal of success by Rudnick and Spratt in carrying out studies in the organization of the early chick blastoderm. Culturing tissues *in vitro* affords opportunity for analyzing the innate capacities of parts of an organism in complete isolation. One great advantage lies in the fact that the living isolate can be kept under continuous, direct observation. Thus the movements of cells, the formation of tissues, *etc.*, can be followed. Prolonged survival of the tissues, however, is not possible. The early chick blastoderm can develop rather normally for a period of about two to three days. In general, the surface resting on the plasma clot is apt to be retarded as compared with the free side. Therefore, as originally pointed out by Waddington, development of the pre-somite blastoderm diverges somewhat from the normal scheme of growth and differentiation.

Extensive studies of whole chick blastoderms and transected portions have shown that, in general, the structures differentiating in the explants originate from the germinal region which would normally give rise to them, *i. e.*, they differentiate according to their prospective value. This does not necessarily hold for chorio-allantoic grafts of similar stages and regions. There are many instances in which grafts of early stages indicate the release of a number of potencies far exceeding the prospective normal fate or value of the material transplanted. For example, mesectoderm may yield liver, thyroid, gut, and respiratory epithelium (Rudnick, 1935). It would appear also that the *in vitro* method is somewhat more favorable than the chorio-allantoic grafting method for testing morphogenetic potencies in the early, whole blastoderm. Very small pieces of the blastoderm, however, do not differentiate *in vitro*. Rudnick (1935; 1938 a, b, c) has used both methods separately and in combination in her studies on the organization of the early blastoderm and the regional restriction of its potencies during embryogenesis. She has done more than any other investigator towards resolving certain

contradictions that have arisen in results from the use of the two methods of isolation.

The tissue culture method has been successfully applied to a study of the origin of the primitive streak and its significance in the development of the chick—a problem that has attracted the interest of many investigators for years. The recent work of Spratt (1946), in which he followed with great exactness the movement of carbon particles placed on the surface of pre-streak blastoderms cultivated *in vitro*, has given an answer to many of the questions that have arisen concerning the origin, growth, and regression of the primitive streak. The information has been conveniently summarized in a coherent series of diagrams (*loc. cit.*).

The inductive capacities of the primitive streak have been tested by Waddington (1933; 1934) and his co-workers in a series of experiments in which portions of the streak and its derivatives were introduced into the explanted blastoderm. While the transplants continued to self-differentiate, the host ectoderm in contact with them often gave rise to more or less perfect medullary formations. Ectoderm of widely varying regions of the early blastoderm responded to the inductive stimulus. As in amphibians, the stimulus has proved not to be species-specific. Duck primitive streak will induce axes in chick ectoderm and vice versa (Waddington and Schmidt, 1933), and there is some evidence that the primitive streak of the rabbit has inducing capacity for the ectoderm of the chick (Waddington, 1936). In spite of the technical difficulties that would have to be overcome to repeat these experiments *in ovo*, it would nevertheless be of the greatest interest to establish the occurrence of these reactions and to follow their course further in normal development. Some progress has already been made in this direction. Alexander (1937), in his studies on lens induction in the chick, succeeded in transplanting optic vesicles or parts of optic cups to various positions beneath the ectoderm of embryos in early somite stages, without removing the blastoderm from the egg.

The technique of tissue culture has proved to be especially well adapted for investigating the general problem of nutrition in the early chick embryo. Having established that the chick, unlike some other embryos, is entirely dependent upon an exogenous supply of nutrients and, further, that it can develop rather normally for the first few days on various synthetic media of known constitution, Spratt (1949; 1950), has been able to approach for the first time a number of basic problems concerning the specific nutritive requirements for the growth and differentiation of the early embryo. By cultivating early blastoderms on media containing known concentrations of specific carbohydrates, Spratt has been able to show, for instance, that different tissues and organs have different carbohydrate requirements for their morphogenesis and differentiation. This method of studying the developmental processes of the embryo under well-controlled nutritive environmental conditions is of particular interest and promise.

The opportunities afforded by the tissue culture technique have also been utilized to study the differentiation of various organ primordia. The work of Fell (1939) on the origin and development of the avian sternum and the

beautiful experiments of Hardy (1949) on the development of mouse hair serve admirably to illustrate what can be accomplished by this method when handled properly and expertly.

Transplantation to the Coelom of the Chick Embryo. The method of transplanting embryonic tissues to the coelomic cavity of the chick embryo was introduced relatively recently by Hamburger (1938) for studies on the differentiation of the limb bud of the chick. This site has proved to be extremely favorable for the growth and differentiation of all types of embryonic tissues. Isolates are introduced into the coelom through a small opening made in the somatopleure just posterior to the entrance of the large yolk sac vessels. The most ideal time for the operation is at approximately two and one-half days of incubation, when the posterior portion of the embryo is lying flat on the yolk and is not yet enclosed by the amnion. The transplants attach themselves readily to the coelomic wall or to the mesenteries and soon become well vascularized by the blood vessels of the host. The grafts may continue their growth for a long period of time, 17 to 18 days, or even beyond hatching. This long growth period has been of great advantage, for example, in growing pigment-free skin for grafting to newly-hatched host chicks (Rawles, 1944; 1945). In addition to the long period of growth of the grafted tissue, another advantage lies in the fact that the coelom permits undisturbed expansion; hence normal morphogenesis. This is especially evident in the case of rapidly expanding primordia, such as limb buds and optic vesicles.

The superiority of the intra-coelomic environment for tissue growth and differentiation may be illustrated best by comparing the results obtained from its use with those obtained from growing the same tissues under other environmental conditions. Since the early limb buds of the chick have been studied by all of the principal isolation methods—*in vitro*, on the chorio-allantois and in the embryonic coelom—they will serve as a good example. Strangeways and Fell (1926), using the *in vitro* culture method, found that muscle and bone did not develop in the explanted limb bud. Epidermis and cartilage, however, differentiated remarkably well. Although there was some "axial arrangement" of the cartilages, the gross morphology by no means approximated that of the normal limb. The studies of Murray (1926) and E. A. Hunt (1932) showed that on the chorio-allantois histological differentiation of all of the limb elements, skin, muscle, cartilage, bone, could take place in a grafted bud, but the resulting limb was never completely normal in size or shape. Both of these investigators noted a considerable capacity for self-differentiation of the skeletal parts. Typical limb cartilages could be identified in most of the grafts. The best cases showed rather complete skeletal parts, more or less distorted and curved, but nevertheless typical in their relative size and arrangement. Hunt was able to identify some grafts as definitely wing or leg in spite of the fact that the normal relations between musculature and skeletal parts were rarely preserved. Using primordia of the same developmental stages, Hamburger (1938) discovered that limb buds which developed in the coelom were far more normal and perfect than those which developed on the chorio-allantois. In fact,

beautifully normal wings and hind-limbs were obtained in a large percentage of cases. The remarkable degree of morphological and histological perfection attained shows clearly that the environment of the coelom furnishes optimal conditions for the expression of the intrinsic developmental capacities of the transplanted limb bud. Such findings are not peculiar to the wing or leg primordia but hold also for other organ-forming tissues. It should be emphasized, however, that any one method furnishes valuable information regarding the developmental capacities of an isolate under particular conditions. In the case of the limb primordia, each method employed showed in a specific manner that the limb was not dependent upon innervation for its differentiation and that the early bud was a self-differentiating system. Even the most abnormal limbs were not interpreted as being due to any specific restriction of the limb-forming potencies, but rather to abnormal spatial conditions of the membranous environment.

While the intracoelomic technique does have certain definite advantages, it also has its limitations. It cannot be applied, for instance, to a study of the axial differentiation of a limb. Transplants cannot be definitely oriented in the coelom. This difficulty may be overcome by transplanting the primordia directly to the outer body wall. By doing so, Hamburger (1938; 1939) was able to show that the antero-posterior and dorso-ventral axes were already definitely determined in both wing and leg buds at the earliest stages tested (25 somites).

The experiments of Hamburger with the limb bud led to an investigation by Rudnick (1945) of the limb-forming potencies of the early blastoderm. It was discovered that definite areas of the blastoderm of early somite stages in the vicinity of the sinus rhomboidalis already possessed specific properties for limb differentiation. A certain percentage of coelomic grafts from these areas differentiated as recognizable limbs. Limbs as such have not been identified in chorio-allantoic grafts or explants of blastoderms of similar stages, although the tissue components of limbs, cartilage, bone, muscle, and skin differentiate readily. These isolation methods have been more conducive to histological differentiation or, at most, to small-scale primitive organogenesis rather than to the differentiation of large or complex organs, such as limbs.

Other methods have also been applied to the chick limb bud for the study of particular developmental problems. Saunders (1947) tested the regulatory capacities of wing buds at specific developmental stages by excising rectangular blocks of ectoderm plus underlying mesoderm and substituting similar tissue blocks from the leg bud. Later, he also (1950) applied carbon marking methods in conjunction with extirpation experiments towards mapping the prospective value of the various wing tissues at successive limb bud stages. He was able to show that the materials for the future wing parts are laid down in proximo-distal order and in their definitive spatial pattern, apparently by a specialized growth zone in the apical region of the bud.

The recent studies of Wolff (1947) serve well to show how the method of intracoelomic grafting can be applied to the study of a fundamental problem such as the time when the developing sex glands of the chick begin to produce

sex hormones. By placing grafts of gonad tissue, isolated at various stages after the onset of sex differentiation, into the coelom in juxtaposition with the gonad primordium of the host embryo, he was able to alter the normal course of sex development of the host, when graft and host were of opposite sex.

About ten years ago, it was found that embryonic mouse tissues would grow and differentiate quite normally in the coelomic cavity of chick embryos (Rawles, 1940). This finding led to a systematic attack of the problem of pigment cell origin for the first time in a mammal. By transplanting isolated portions from various axial levels of potentially pigmented embryos, at successive stages in their development (4-40 somites), to the embryonic chick coelom, it was possible to show that only those isolates containing the neural crest or its migratory cells can produce melanin pigment in the grafts (Rawles, 1947). The fact that skin and hairs, structurally normal but entirely pigment-free (white), developed regularly in grafts from which the neural crest was excluded, led to the conclusion that skin ectoderm and its derivative, the hair, have no inherent capacity for autonomous melanin pigment formation. Since hair development and melanin granule formation occur relatively late in embryonic development, the long period of growth in the coelom meets very satisfactorily the time requirement which is absolutely necessary for such pigmentation studies.

Various isolation and transplantation methods have been applied to the study of the differentiation of a variety of embryonic mammalian tissues. The sites which have been most frequently used *in vivo*, other than the chorio-allantois already mentioned, are the omental bursa of the adult rabbit, the kidney capsule of the adult rabbit and rat (Waterman, 1932; 1936), and the eye chamber of the adult mouse (Runner, 1946; Grobstein, 1951). All of these methods have contributed information in showing that, in the mammalian embryo, as in other vertebrate forms studied, primordia of organs, once they are morphologically indicated, can maintain themselves and undergo a certain degree of progressive differentiation in a foreign environment. Since the embryonic chick coelom has proved to be an extraordinarily favorable site for the growth and differentiation of embryonic mouse tissues, there is good reason to believe that the intra-coelomic grafting method could be applied successfully to other mammalian tissues and to problems of mammalian development in general. The embryonic chick coelom might, for instance, even be useful as a site for the development of mammalian eggs. The tissue culture methods used so far would seem inadequate for the study of segmenting stages, although they have proved much more adequate for the study of later embryonic stages (Nicholas and Rudnick, 1934).

Isolation and Transplantation Methods Applied to the Analysis of the Development of Melanin Pigmentation Patterns. The foregoing account has been concerned primarily with the use of transplantation methods for elucidating problems of early embryonic organization and the progressive differentiation of organs. In the present section, an attempt will be made to show how the various isolation and transplantation techniques have been used to make a step-by-step analysis of a developmental problem of quite

different nature, namely, the development of melanin pigmentation patterns in the feathers of birds.

The first step was to establish the origin of the melanin pigment-forming cell, the melanophore, from the neural crest. This was done by testing the pigment-forming capacity of a great variety of tissues of pigmented fowl and other bird embryos, isolated from various axial levels at successive stages in development. As many as five different methods were used: (1) *in vitro* culturing (Dorris, 1938); (2) intracoelomic grafting (Eastlick, 1939; Ris, 1941); (3) chorio-allantoic grafting (Ris, 1941); (4) grafting directly to the limb buds (Dorris, 1939; Willier and Rawles, 1940; Ris, 1941); and (5) grafting to the outer body wall or flank (Eastlick, 1939). From the results of these experiments it was conclusively demonstrated that only those isolates known to contain the neural crest, prospective neural crest, or cells migrating from the neural crest are capable of producing melanophores. Isolates containing skin ectoderm and mesoderm but no crest cells regularly produced feathers which were structurally normal but white, since no melanin pigment granules could be synthesized in the absence of melanophores.

The experiments cited above, particularly the intracoelomic grafting experiments and those in which neural crest cells were grafted directly to the limb bud, in addition to establishing the origin of melanophores, established another important fact, namely that melanophores reach their definitive locations in the feather germs by an extensive migration occurring early in development when the melanophores are in their colorless, undifferentiated, melanoblast stages. It was found, for example, that melanoblasts have already reached the limb buds by the fourth day of incubation, before the feather germs arise and before there is any visible evidence of melanin pigmentation.

Having, then, established the origin of the precursor melanophores and the time when they are located in certain regions, such as the limb buds, and furthermore that feathers can develop normally in the complete absence of melanophores, the next step was to analyze the respective roles of the melanophore and the feather germ in the formation of the definitive color pattern. This was done by combining melanoblasts of individuals of breeds exhibiting a specific type of color and pattern with feather germs of individuals exhibiting a different color and pattern. Again, a number of grafting methods were employed singly and in combination: (1) grafting melanoblasts from various regions of a donor embryo to the early wing bud of a host embryo, prior to the entrance of the host's neural crest cells, and raising the chicks to adulthood to observe the color patterns of successive generations of feathers (Willier and Rawles, 1940; Rawles, 1939); (2) introducing melanoblasts from the parietal lining into areas of wing skin and wing feather germs [this involved two steps: (a) grafting the early wing bud to the coelom of a host embryo of a pigmented breed where it would become invaded by melanoblasts from the host parietal lining, and (b) grafting the wing skin with its pigmented down plumage, dissected from the intracoelomic wing graft obtained by step (a), to the saddle region of a newly-hatched host chick of another breed] (Rawles, 1945); (3) introducing melanoblasts

from the saddle region of a newly-hatched chick into an area of pigment-free wing skin of similar age obtained (a) by grafting the early wing bud to the embryonic coelom of a White Leghorn where it will grow and differentiate without being invaded by melanoblasts, and then (b) grafting the pigment-free wing skin and down feathers, dissected from the intracoelomic wing graft obtained in (a), to the saddle region of a newly-hatched pigmented host chick (Rawles, 1944); and (4) by transplanting the feather papillae between adult individuals (Wang, 1944).

The results obtained from all of these methods, in which many varieties of fowl and other birds were used, have been consistent in showing that melanophores produce their specific color and pattern in homologous feathers of varieties normally exhibiting an entirely different color and pattern, regardless of the method of introducing them into foreign feather germs and regardless of their immediate source. Melanophores of Barred Plymouth Rock origin, for example, produce always a barred pattern when introduced into feather germs of any other breed, White Leghorn, New Hampshire Red, Black Minorca, *etc.* The specific type of barring characteristic of the various body regions, the width of the bars, for instance, is dependent, however, upon the tract location of the feather germ in which the melanophores function. In other words, a barred wing pattern is produced only in wing feather germs, a breast pattern only in breast feather germs, a saddle pattern only in saddle feather germs, *etc.* The interpretation towards which these experiments and other investigations have led is that color patterns arise through a constant interaction between melanoblasts and their tissue substrates, and to reactions between the melanoblasts themselves. A discussion of the various extrinsic factors, such as hormones, for example, which definitely affect the response of the pigment cells in the formation of color patterns in certain breeds, is beyond the scope of this paper. The reader is therefore referred to recent reviews by Willier (1948) and Rawles (1948). Enough has been said, perhaps, to indicate the value of the use of a variety of experimental methods and their special adaptation to the analysis of a particular problem.

In summary, it may be said that the application of specific methods of isolation and transplantation to the solution of special problems has contributed importantly to our knowledge of the early organization of the embryo and the progressive differentiation and growth of many of its parts. An understanding of the developmental capacities of normal tissues and their reactions, under as wide a variety of environmental conditions as it is possible to impose, is essential to an interpretation of normal as well as abnormal tissue growth.

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TUMOR TRANSPLANTATION TO THE CHICK EMBRYO*

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In 1912, J. B. Murphy¹ reported that mouse and rat tumors would grow when explanted to the chorio-allantoic membrane (CAM) of the chick embryo, and that these tumors could be maintained by continuous passage from egg to egg. The year previously, Rous and Murphy² demonstrated that the Rous chicken sarcoma, transplanted to the CAM, would induce tumors of the membrane. Since these original reports, tumors from chickens, ducks, mice, rats, rabbits, guinea pigs and man have been explanted in the fertile egg, by the CAM, yolk sac, intra-embryonic and intravenous routes. The developing chick embryo has no defense against the growth of heterologous tissue, and it will supply blood vessels and connective tissue for the growth of these tumors.

The following methods are examples of different procedures used in tumor transplantation:

*Chorio-allantoic Membrane Route.*³ The eight-day embryo is used. A window is cut in the shell over the vascular area, and a tumor fragment, 1-2 mm. in size, is placed on a large vessel. The shell opening is sealed with Scotch tape, or with a coverglass and paraffin. The egg is incubated at 99-100° C.,⁴ and the embryo is sacrificed at 17 to 18 days, and the tumor removed.

*Yolk Sac Route.*⁵ The four-day embryo is used. Tumor tissue is prepared by adding one cc. of tumor suspension to four cc. of a 1:1 mixture of egg white and saline. One-half cc. of this tumor preparation is injected into the yolk sac, and the embryos sacrificed at 17 days.

*Intravenous Route.*⁶ The 11-day embryo is used. The shell is removed over the air sac and the blood vessels exposed. Five-hundredths (0.05 cc.) of the sterile tumor suspension is injected intravenously, and the embryo is sacrificed on the 20th day.

*Intra-embryonic Route.*⁶ Embryos 50-60 hours old (26-34 somites) are used. The embryo is exposed through a window in the shell, and a strip of somatopleure lateral to somites 24 to 32 is removed. A piece of tumor tissue is fitted into place, and the shell opening repaired. The embryos are usually sacrificed six to eight days later.

The behavior of tumors explanted to the chick embryo has been measured by the growth of the tumor, its histological appearance, growth by continuous passage in eggs, viability when transplanted to its original host, and the effect of the growing tumor on the chick embryo. The chick embryo has been used in many tumor studies, and we will summarize some of this work with illustrative material drawn from our own studies.

* This work was supported by grants from the National Cancer Institute of the United States Public Health Service, the Damon Runyon Memorial Fund for Cancer Research, and the American Cancer Society.

Chicken Tumors

Rous Chicken Sarcoma. Rous and Murphy² found that the Rous chicken sarcoma produced tumors on the CAM and occasionally in the embryo. This work has been confirmed by several workers,^{3, 8, 9} and Bueker⁷ has described sarcomas in the chick embryo following the intracoelomic implantation of the Rous chicken sarcoma. Keogh,¹⁰ using cell-free filtrates of Rous sarcoma, obtained both ectodermal and mesodermal proliferative lesions of the CAM. Of great interest was Milford and Duran-Reynals¹¹ observation that the intravenous or intra-embryonic injection of the Rous sarcoma tissue or cell-free extracts would induce a fatal hemorrhagic disease of the embryo without evidence of neoplasia.

In our own work we obtained Rous sarcoma growing in a chicken from Duran-Reynals. Small fragments were inoculated onto the CAM of the eight-day embryo. The embryos almost invariably died seven to eight days after transplantation. At this time large tumors were found on the CAM, sometimes weighing up to five grams. The tumors covered a large area of the membrane, and there was a small necrotic dimple at the site of implantation. The mass of the tumor was hanging downward on the under side of the CAM. An involved membrane is shown in FIGURE 1a. Microscopically, the tumor was composed of loosely arranged cells with occasional areas of condensation. The cells were of varying size, and mitotic figures could be found easily (FIGURE 1b). Death of the embryo was apparently due to a massive hemorrhage, for a large amount of free blood was found on the CAM, in the yolk sac, or in the abdomen. Throughout the embryo, but particularly in the liver, were small hemorrhagic nodules (FIGURE 1c), and it is likely that rupture of one of these nodules was the cause of death. Embryonic growth and development in the presence of the Rous sarcoma otherwise appeared to be normal. Tissues from the embryo, removed three days or later after the tumor was inoculated on the CAM, produced typical sarcomas when transplanted to the CAM of other eight-day embryos. This indicated that virus was spread widely throughout the embryo within three days after the Rous sarcoma was transplanted to the CAM. Cell-free filtrates of the Rous sarcoma induced pox-like lesions when injected on the CAM, and also caused the hemorrhagic syndrome. When the Rous sarcoma tissue was irradiated *in vitro* with 25,000 r, a dose presumably sufficient to destroy all the cells, and fragments then transplanted to the CAM, tumors as large as those produced by untreated tissue developed. It is reasonable to believe that these tumors were produced by the virus acting directly on the cells of the chick embryo and not from the outgrowth of irradiated explanted cells. Since the cell-free filtrate produced small pox-like lesions on the CAM in contrast to the large tumors resulting from the irradiated tissue, this would indicate that a great deal of virus or a protective substance is lost by ultrafiltration. Rous sarcoma tissue has produced yolk sac tumors following yolk sac injection, and the embryo also developed the hemorrhagic syndrome.

Fowl Leucosis RPL-12. Fowl lymphomatosis is due to a cell-free agent, which has a long latent period and may be transmitted through the egg to

the developing embryo. While there have been suggestions that some strains of fowl leucosis can induce tumors rapidly on inoculation, it appears likely that rapid tumor growth is due to the transplantation of viable cells.¹² Olson¹³ described a transmissible lymphoid tumor, RPL-12, and this tumor

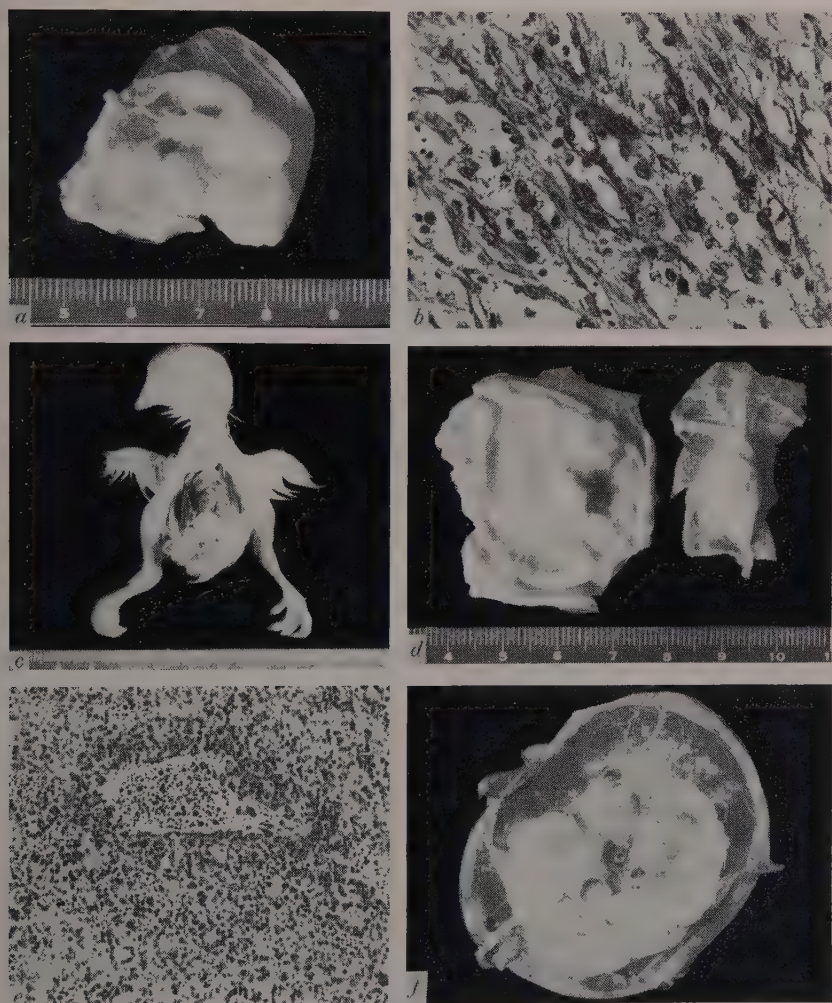


FIGURE 1. The effects of the Rous sarcoma and fowl leucosis RPL-12 on the CAM and the chick embryo. (a) Large nodular Rous sarcoma on the CAM of the 15-day embryo; tissue had been implanted at eight days. The embryo was sacrificed within 24 hours of death. (b) Microscopic section of the Rous sarcoma growing on the CAM; note the pleomorphism and the loose arrangement of cells. (c) Embryo, sacrificed at 15 days of age, which received an implant of Rous sarcoma on the CAM at eight days. The liver contains numerous small, hemorrhagic nodules, and one is also evident in the wall of the intestine. This embryo would have died of an acute hemorrhage within the next day. (d) Two chorio-allantoic membranes of 15-day embryos, which received an explant of leucosis RPL-12 cells at eight days. There are large central nodules, with the radiating white blood vessels infiltrated with leucosis cells. (e) Section of the liver of a 15-day embryo, which received a CAM transplant of RPL-12 leucosis at eight days. A cuff of leucosis cells surrounds the vein, and there are numerous leucocytes in the circulating blood. The liver parenchyma is also infiltrated with the leucosis cells. (f) CAM from 15-day embryo, implanted at eight days, with a mixed suspension of Rous sarcoma and leucosis RPL-12 cells. The entire membrane is involved with nodules, and at the lower edge are seen the large white blood vessels, infiltrated with leucosis cells.

was shown to contain lymphomatosis virus. Chickens immunized against the RPL-12 leucosis, when inoculated with RPL-12 leucosis cells which failed to grow, subsequently developed, after a long interval, a high incidence of lymphomatosis.¹⁴

We obtained the RPL-12 strain of cells from Cottrall, whose group had grown them successfully in the chick embryo. Pierce¹⁵ also was able to grow leucosis cells in the chick embryo. Fragments of RPL-12, when explanted to the CAM of the eight-day embryo, produced a large, pale nodule with white radiating cords extending over the CAM (FIGURE 1d), and, on histological examination, the walls of the blood vessels were massively infiltrated with leucosis cells. These cells apparently grew out from the implant, extended along the blood vessels to the embryo, and the embryos almost invariably died seven to eight days after inoculation. The embryo at death had a greatly enlarged liver and a slightly enlarged spleen. The liver showed massive infiltration with leucosis cells, particularly around the blood vessels, and there was an obvious increase in lymphoblasts in the blood vessels (FIGURE 1e). Yolk sac inoculation with a suspension of leucosis cells also produced the disease. Fragments of the liver from embryos dying of leucosis, when inoculated on the CAM of other eight-day embryos, produced active leucosis in the embryo. If the leucosis nodule were irradiated *in vitro* with 5000 to 7000 r, it no longer produced active growth on explantation to the chick embryo; which is evidence that the disease, as it is produced in the chick embryo, is due to the growth of viable transplanted cells.

Suspensions of Rous sarcoma and RPL-12 cells were mixed together, incubated for one hour, and then inoculated on the CAM. Each suspension seemed to produce its characteristic growth, without interfering with the other (FIGURE 1f).

Spontaneous¹⁶ and carcinogen-induced¹⁷ avian tumors have been explanted to the CAM and growth was either transient or unsuccessful.

Mouse Tumors

Mouse tumors have been used most commonly for tumor transplantation in eggs, and all routes of inoculation have been used.

Chorio-allantoic Membrane Route. Stevenson¹⁸ reported in detail on the growth of the Erlich and Crocker sarcomas and the Twort carcinoma on the CAM; he suggested that each tumor grew at approximately the same rate in the egg as in the mouse. Jacoby *et al.*¹⁹ grew a carcinogen-induced sarcoma on the CAM. We have studied, at the Sloan-Kettering Institute and the Jackson Memorial Laboratory, the growth characteristics of 27 different mouse tumors when explanted to the CAM of the eight-day embryo. FIGURE 2a shows the gross appearance of an osteogenic sarcoma ten days after inoculation and FIGURE 2b shows a low-power view of the microscopic appearance of a similar type of tumor. A low-power view of another type of tumor, the EO771, growing on the CAM, indicates how each tumor tends to retain its characteristic histological appearance when grown in the chick embryo (FIGURE 2c). Each tumor was carried for at least three continuous egg passages when possible (each tumor was on the CAM nine to ten days

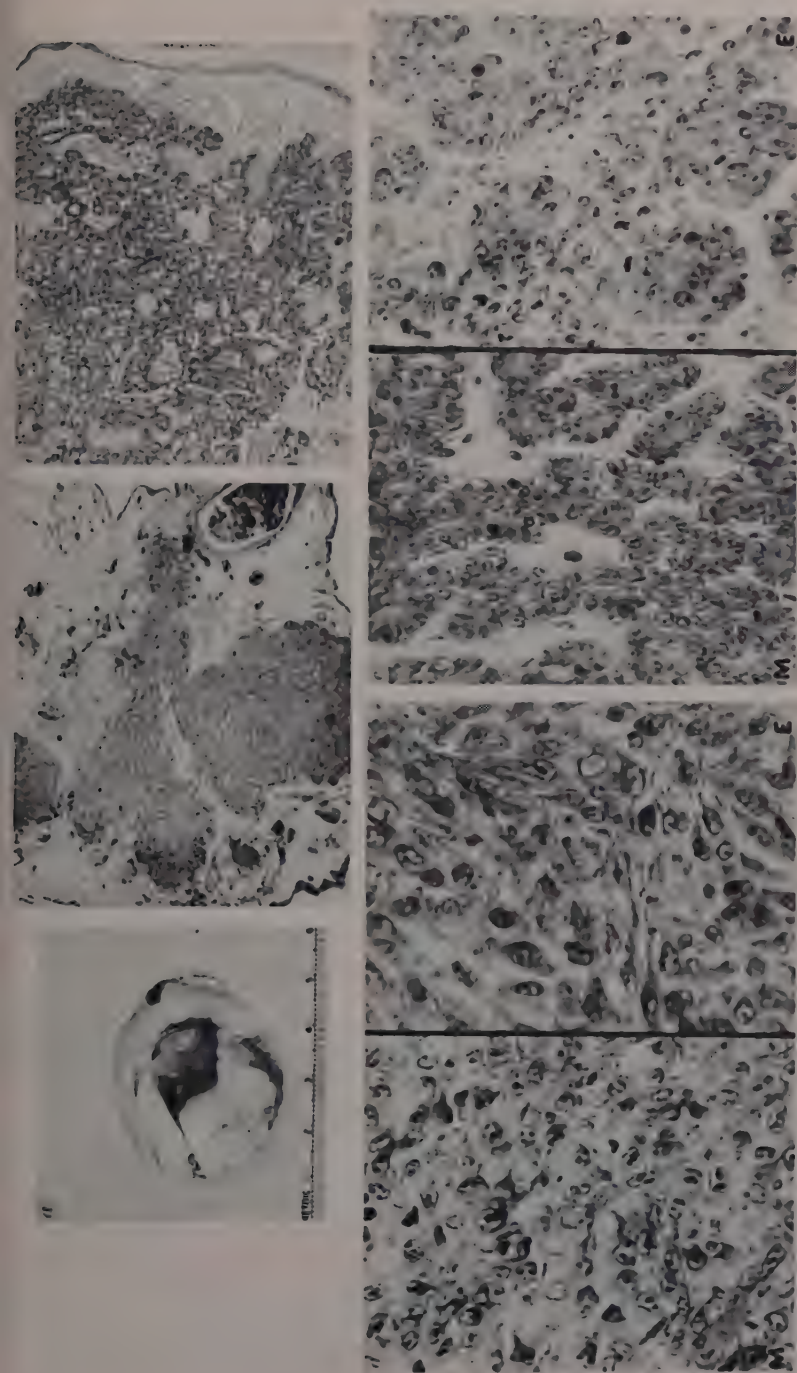


FIGURE 7. Appearance of mouse and egg-grown mouse tumors. A: First egg passage of the osteogenic sarcoma (Ridgway) growing on the CAM of the 15-day embryo. The tumor is shown in the shell. Tumor was explanted to CAM of eight-day embryo. B: Osteogenic sarcoma (Wagner) growing on the CAM of the 16-day embryo. The thickening membrane contains large nodules of soft mouse tumor and serous cavity blood vessel. There is also an area of epithelial hyperplasia, a reaction occasionally seen on a CAM implanted with tissue or a forelet body. C: Mouse mammary adenocarcinoma (Fogel) third passage growing on the CAM of the 17-day embryo. The cells maintain an alveolar arrangement in the egg as they do in the mouse. D: Mammary carcinoma (spindle cell) 100% microscopic section of tumor growing in mouse (M) and after three passages in egg (E). Cells of the egg-grown tumor are larger, and the spindle cell arrangement is maintained. E: Mammary adenocarcinoma (H712b), tumor growing in mouse (M), and after two passages in egg (E). This tumor retains its alveolar structure in the egg, and mitotic figures are frequent.

during each passage), and some tumors were passed many more times. The sarcoma 180 was maintained by continuous passage for one year on the CAM. After each egg passage the tumors were assayed for viability in susceptible mice and examined microscopically. As a result of our observations on the growth characteristics of these tumors, they have been tentatively divided into six groups (TABLE 1). While the rate of growth of the tumor in the mouse is of great importance in determining the growth of the tumor in the embryo, other factors are obviously involved.

Group A. Group A is composed of the mouse leukemias and lymphosarcomas. These tumors grew rapidly on subcutaneous transplantation in mice to form large local tumors, and disseminated to produce generalized leukemia. They showed no active growth when explanted to the CAM, although some viable cells occasionally were present at the end of the first egg passage, as proved by successful transplantation to mice. Of the tumors tested, the only exception was lymphosarcoma P3269, which showed slight growth in the egg. The cells survived for two egg passages.

Leukemic cells were explanted to the CAM in conjunction with another rapidly growing tumor from the same strain of mouse (Patterson lymphosarcoma and Ma387). These two tumors also were inoculated into the same subcutaneous site in mice, and the resultant mixed tumor explanted onto the CAM, but growth of the leukemic cells was not enhanced in either instance. Thiersch²⁰ was unable to transmit mouse leukemia in the chick embryo by injecting cell suspensions intravenously. Lymphomas apparently failed to grow on heterologous transplantation to the anterior chamber of the eye.²¹ It is of interest that the RPL-12 fowl leucosis, in contrast to mouse leukemia, grew actively in the embryo. The poor growth of the mouse leukemia and lymphosarcoma in the chick embryo is an unexplained and provocative problem.

Group B. Group B consists of tumors which grew slowly in the mouse and showed a similar slow rate of growth on the CAM, so that the tumors rarely survived the first egg passage. It is assumed that these tumors do poorly in the egg because they have an inherently slow rate of growth. This is probably also the case in human tumors, which may survive the first passage in the egg, but do not grow rapidly enough to permit continuous egg passage.

Group C. Group C consists of tumors, chiefly mammary adenocarcinomas, which grew at a moderate rate in the mouse and also in the egg, so that the tumor survived for only two or three passages on the CAM (FIGURE 2d, e). This decrease in growth rate of the tumor in the egg may be due to the disturbance of the slowly growing tumors, incidental to their removal when the tumor cells were transplanted at nine- to ten-day intervals.

Group D. Group D contained five sarcomas, which apparently could be carried for an indefinite period by continuous passage in the egg, and these tumors showed approximately the same growth rate in the mouse and egg (FIGURE 3a, b). The tumors in this group are the most satisfactory ones available for CAM transplantation in our experience.

Group E. Group E is represented by a single tumor, C1300, presumably

TABLE 1

SUMMARY OF THE RESULTS OBTAINED WITH TWENTY-SEVEN MOUSE TUMORS EXPLANTED TO THE CHORIO-ALLANTOIC MEMBRANE OF THE CHICK EMBRYO

Tumor		Susceptible mouse strain	Rate of growth in mouse after transplantation			Characteristics of growth in eggs		
Name	Type		First palpable	20 x 20 mm. size	Death of mouse	Size of nodule, first pass. mm.	Growth, second passage or more	Bioassay, mice
			(weeks)					

Group A

Tumors growing rapidly in mice, but showing slight or no growth in eggs

C1498	Myelo. leukemia	C57B/6	$\frac{1}{2}$	1-2	2-3*	2 x 2	none	negative
L1394	Chloro. leukemia	AKM	1	2	3*	2 x 2	none	occ. positive, 1st pass. only
P1534	Lymph. leukemia	DBA 2, 3, 4	$1\frac{1}{2}$		2*	1 x 2	none	occ. positive, 1st pass. only
H2876	Lymph. leukemia	C/58	1	1-2	4-5*	2 x 2	none	occ. positive, 1st pass. only
Patterson	Lympho-sarcoma	AK/M	1	2	3-4*	3 x 3	none	occ. positive, 1st pass. only
6C3HED	Lympho-sarcoma	C3H/An	$\frac{1}{2}$	1-1 $\frac{1}{2}$	2	3 x 4	none	occ. positive, 1st pass. only
P3269	Lympho-sarcoma	RIII	1	1-1 $\frac{1}{2}$	4	5 x 5	small	positive, 2 pass only

Group B

Tumors growing slowly in mice, and also in eggs

Harding-Passey S91	Melanoma	most strains	4	8-9	12-15	1 x 2	none	negative
	Melanoma	DBA	3-4	6	9-10	1 x 2	microscopic evidence for 3 passages	positive, 1st pass. only
AK	Mammary adenoca.	AK/M	3-4	5-6	7-9	1 x 2	none	negative
Wagner	Osteogenic sarcoma	AK/M	1-2	4-5	6-7	3 x 4	none	positive, 1st pass. only

Group C

Tumors growing at a moderate rate in mice and also in eggs, so that tumors were smaller on successive egg passage

E0771	Mammary adenoca.	C57	$\frac{1}{2}$ -1	2-3	6-7	10 x 12	mod. decrease growth, 3 pass.	positive for 3 pass. tested
H2712b	Mammary adenoca.	C3H	2	3-3 $\frac{1}{2}$	4-4 $\frac{1}{2}$	7 x 9	mod. decrease growth, 3 pass.	positive for 3 pass. tested
15091a	Spindle cell ca.	A	1	1 $\frac{1}{2}$ -2	4-4 $\frac{1}{2}$	5 x 5	mod. decrease growth, 3 pass.	positive for 3 pass. tested
Street	Mammary adenoca.	St	2	5	8-9	2 x 3	grew for 2 pass.	positive for 2 pass. tested

* Died of generalized leukemia.

TABLE 1 (Continued)

Tumor		Susceptible mouse strain	Rate of growth in mouse after transplantation			Characteristics of growth in eggs		
Name	Type		First palpable	20 x 20 mm. size	Death of mouse	Size nodule, first pass. mm.	Growth, second passage or more	Bioassay, mice
			(weeks)					
Group C (Continued)								
C ₃ HBA	Mammary adenoca.	C3H An	1-1	1-2	3-4	5 x 5	slow growth, viable cells on 2 pass.	positive, 1st pass. only
H2712a	Mammary adenoca.	C3H	2	3-4	6-7	3 x 3	grew on 1st pass. only	positive 1st pass. only
dbrB	Mammary adenoca.	DBA/1	1	1-2	3	3 x 3	grew on 1st pass. only	positive, 1st pass. only
Group D								
Tumors growing at a uniform rate on egg passage								
Sarcoma 180	Cuboidal cell	most strains	1-1	2-3	3-4	6 x 8	uniform rate	positive, 30 pass. tested
Sarcoma 1	Spindle cell	most strains	1-1	2	6-7	5 x 5	uniform rate	positive, 3 pass. tested
Sarcoma 37	Spindle cell	C57	1-1	1-2	2-3	10 x 12	only one pass. tested	positive, 1 pass. tested
Ma387	Spindle cell	AK/M	1	3-4	4-5	6 x 7	uniform rate	positive, 8 pass. tested
202L	Spindle cell	C3H/An	1-1	2-3	3-4	4 x 4	uniform rate	positive, 3 pass tested
Group E								
Tumors growing more rapidly in the egg than in the mouse, and growing on continuous egg passage								
C1300	Neuro-blastoma	A	1	2-3	6	20 x 20	continuous rapid growth	positive, 3 pass. tested
Group F								
Tumors showing rapid growth on first passage in the egg, and a marked drop in growth rate on second passage								
Ridgway	Osteogenic	AK/M	1-2	3-4	4-5	10 x 12	no growth or slight, 2nd pass.	positive, 1st pass: rarely positive 2nd
C954	Liver ca.	C57L	1-2	2-3	5	9 x 10	slow growth, but viable cells, 3 pass.	positive, 2 pass.
S637	Cuboidal cell sa.	P/Sn	1	2	4-5	7 x 8	none	positive, 1 pass only

a neuroblastoma, which grew more rapidly in the egg than in the mouse (FIGURE 3c). Some of these tumors weighed two grams after ten days growth on the CAM. This result suggests that there may be factors in the mouse which inhibit the growth of the C1300.

Group F.—Group F consists of three tumors which grew rapidly on the

first egg passage, but then showed a precipitous decrease in growth rate on subsequent egg passage. This was very strikingly evident in the case of the Ridgway osteogenic sarcoma. This tumor grew very well on the first passage (FIGURE 3d), but failed to grow on the second passage. Tumor tissue obtained from the first egg passage and inoculated into mice also grew slowly, but, after a tumor appeared in mice, it exhibited its original rapid growth on re-inoculation to the CAM. The liver tumor C954 (FIGURE 4a) and sarcoma S637 seemed to behave in the same manner. It is possible that these tumors may carry over some factor from the mouse essential for their continuing growth, and this factor is used up during the first passage.

Various combinations of tumors can be grown successfully on the CAM, and FIGURE 4b shows the sarcoma 180 and Ma387, each retaining their characteristic cuboidal and spindle cell appearance, while growing together on the CAM. In our experience, none of the CAM-grown mouse tumors has produced any grossly evident or consistent abnormality in the developing chick embryo.

Yolk Sac Route. Taylor *et al.*⁵ developed the technique of yolk sac transplantation of mouse tumors. While they note that a number of tumors may grow in this environment, the best growth has been obtained with a dba and a C₃H mammary carcinoma. These tumors grew consistently, and averaged one to three grams respectively, when the 17-day-old embryo host was harvested. Twombly and Meisel²² obtained yolk sac growth with mammary carcinomas (755 and RC), but not with the sarcoma 180. Mammary carcinomas have been grown in the yolk sac by Armstrong and Ham.²³ Of considerable interest is the observation, first reported by Heilman and Bittner,²⁴ that continuing passage of certain tumors in the yolk sac would finally render them toxic to the chick embryo.²⁵ Armstrong and Ham²³ reported a specific syndrome appearing in the embryo after ten egg passages of a C₃H and Strain A mammary carcinoma. The affected embryos were small, severely anemic, and the livers and hearts were enlarged. There was no correlation between the size of the tumor growth and the degree of anemia, but the anemia was correlated with the number of egg passages of the tumor. The mechanism of the enhanced toxicity of tumors to the chick embryo on repeated yolk sac passage, or why it has not been obtained with CAM tumor transfers, remains unexplained.

Other Animal Tumors. The experiences with rat and rabbit tumors have been somewhat similar to those with mouse tumors.²⁶ Schechtman *et al.*²⁷ obtained good growth with the Brown-Pearce carcinoma of rabbits by continuous passage on the CAM, and observed no toxicity to the embryo or evidence of intra-embryonic metastases. In our experience, the V2 carcinoma of rabbits failed to grow on the CAM. Smith²⁸ maintained the rabbit fibroma virus on the CAM for 18 passages and membrane edema was the only lesion seen.

Human Tumors

Murphy¹ tried to grow human tumors on the CAM and the results were less successful than with mouse tumors, but, in some cases, the explanted

tumors contained mitotic figures, and a copious blood supply was furnished by the embryo. Hurst, Cooke and McLennan²⁹ transplanted a number of normal and neoplastic human tissues to the CAM, and several of the explanted tissues survived and showed mitotic activity during the first egg passage.

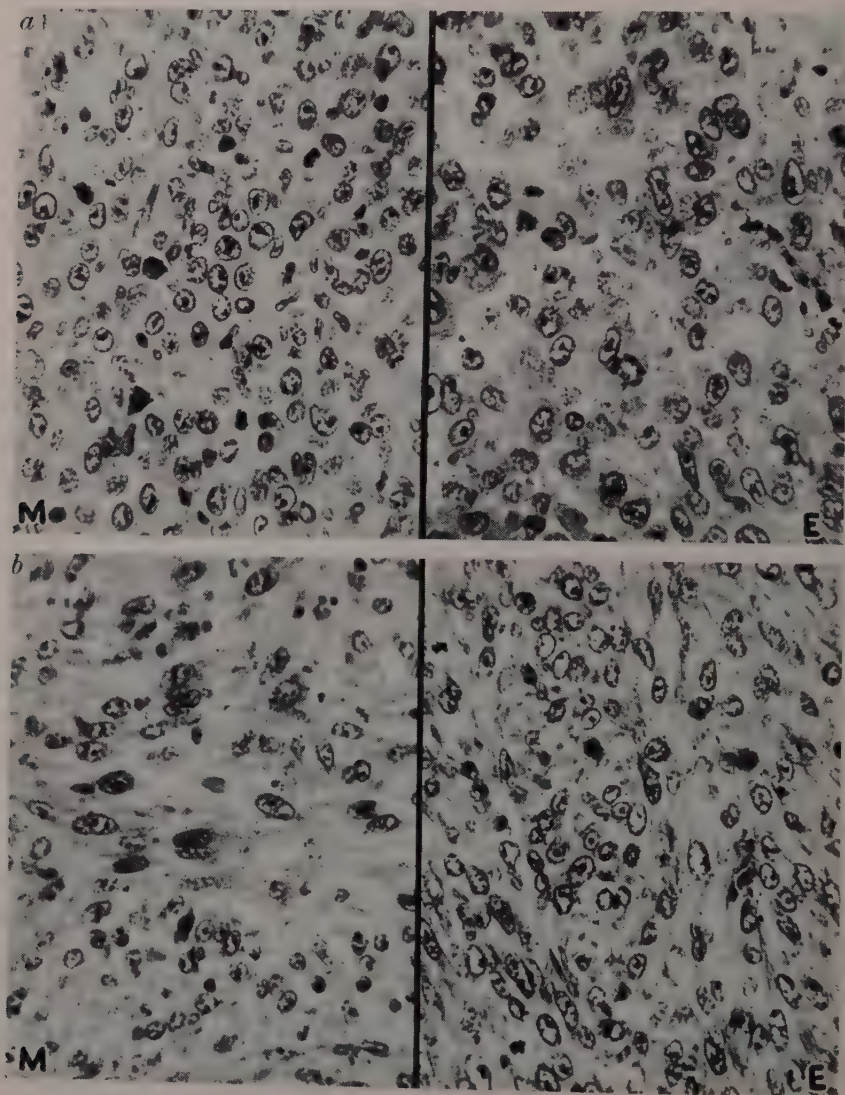


FIGURE 3. Appearance of mouse and egg grown mouse tumors. A. Sarcoma 180: microscopic section of tumor growing in mouse (M), and after eight passages in the egg (E). The cells are larger in the egg-grown tumor. B. Spindle cell sarcoma Ma387: tumor growing in mouse (M), and after four passages in the egg (E). In the mouse and egg, bundles of spindle cells run in different directions. C. C1300 (neuroblastoma): tumor growing in mouse (M), and after three passages in the egg (E). Nucleated erythrocytes are seen in the chick-grown tumor. D. Osteogenic sarcoma (Ridgway): tumor growing in the mouse (M), and after one passage in the egg (E).

During a three-year period, 71 human tumors, freshly obtained by biopsy, have been explanted to the CAM. These tumors included: Hodgkin's dis-

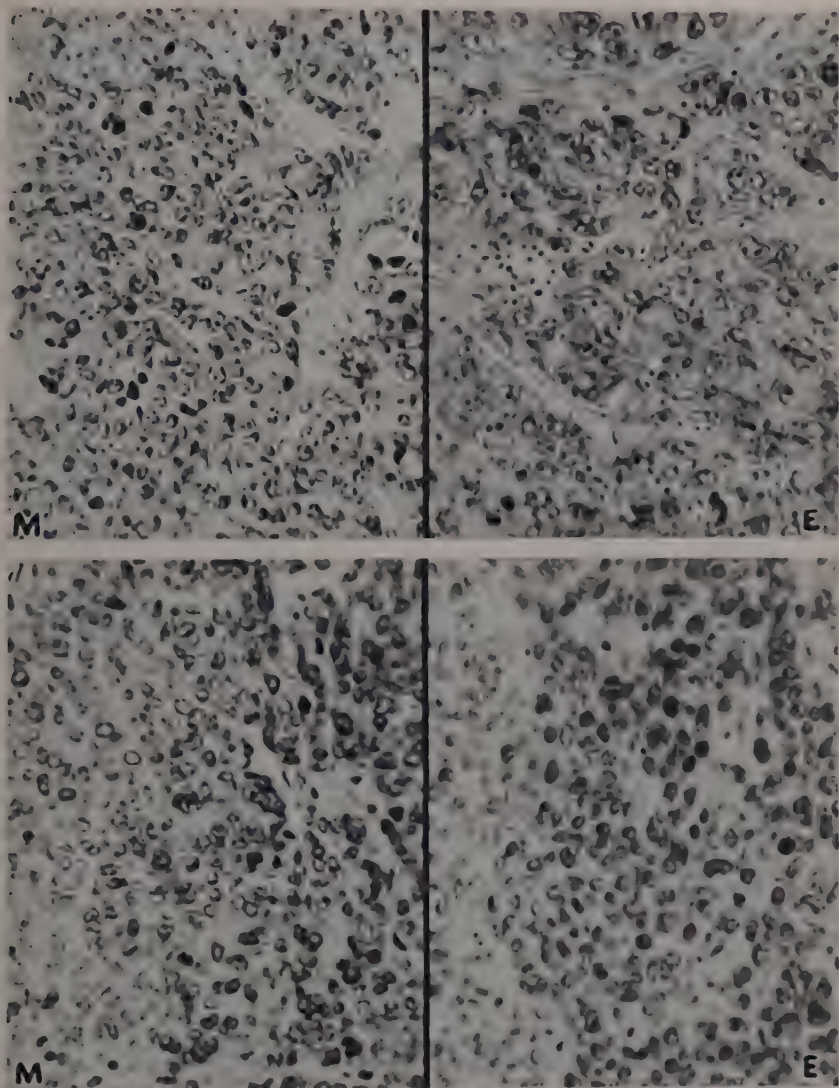


FIGURE 3. continued

ease, 21 cases; lymphosarcoma and lymphatic leukemia, 28 cases; and 22 miscellaneous tumors, including five carcinomas of the lung, four mammary carcinomas and two melanomas. In general, these tumors grew very poorly, and only in a few instances was there evidence of cell survival and mitotic

activity. None of the human tumors could be maintained in the egg beyond the first passage. Several interesting observations were made.

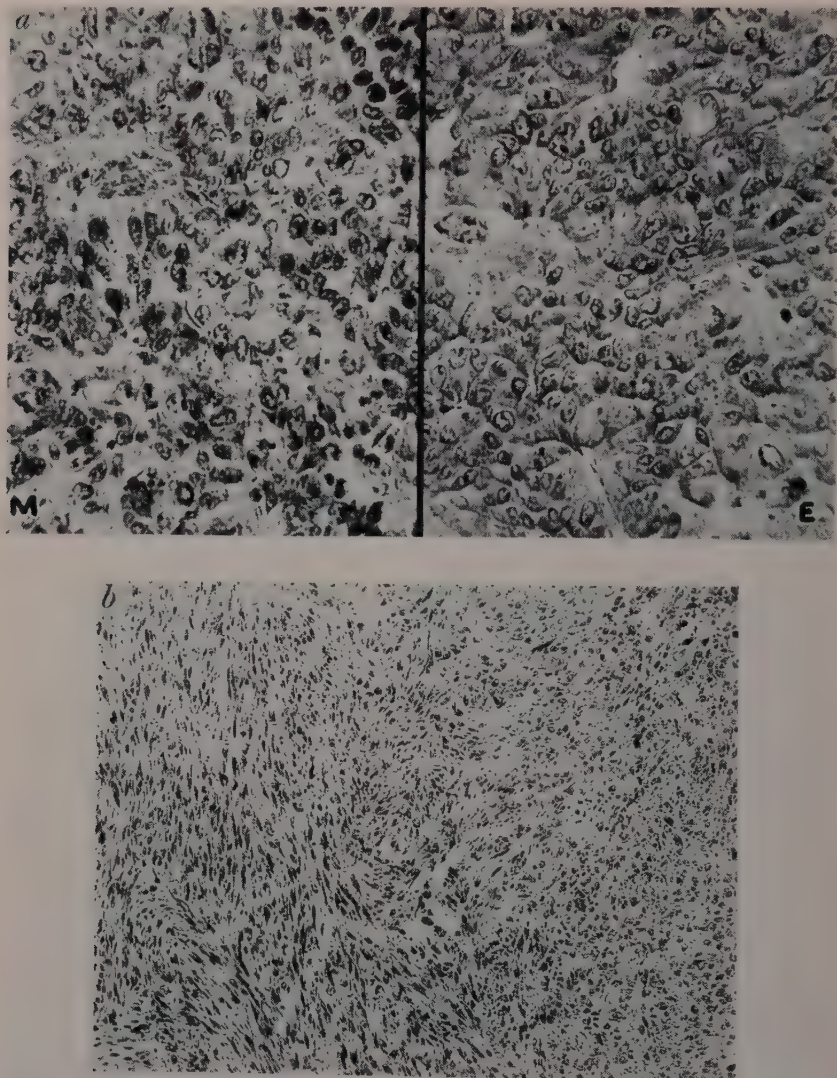


FIGURE 4. Appearance of mouse and egg-grown mouse tumors. A. Liver carcinoma C954: microscopic section of tumor growing in the mouse (M), and after one passage in the egg (E). B. Microscopic section of Ma387 (spindle cells) and sarcoma 180 (cuboidal cells) grown together on the CAM. Each tumor retains its characteristic appearance, and grows separately.

Membrane Edema. Lymph nodes or tissues containing lymphocytes explanted to the CAM produced an edematous disc on the membrane, one to two cm. in diameter, around the explant (FIGURE 5a). This reaction appeared within 24 hours, and subsided usually within three to four days.

Tissues stored in the refrigerator for several days showed a marked decrease in this activity. The effect could not be transmitted from one CAM to another by transfer of the tissue. Since this reaction was not obtained with

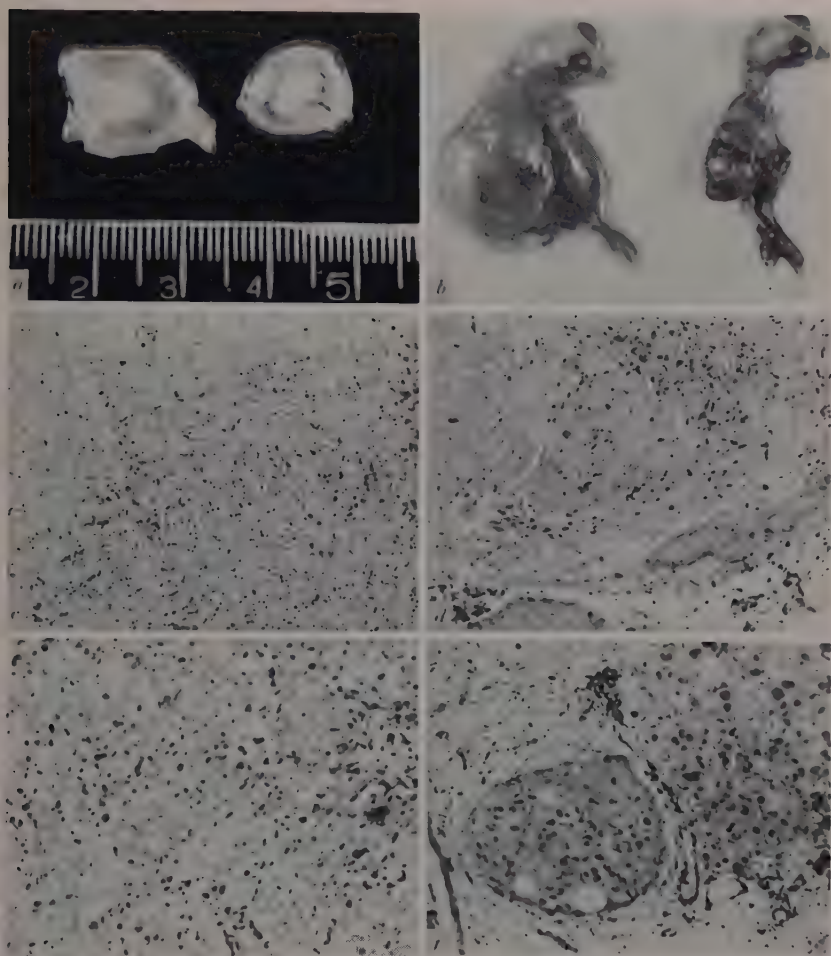


FIGURE 5. Human tumors explanted to the CAM of the chick embryo. A. Two edematous discs surrounding the implants of fragments of lymphatic tissue on the CAM. This reaction is present within 48 hours after the tissue is placed on the CAM of the eight-day-old embryo. B. Edematous embryo produced by the implantation of Hodgkin's disease tissue on the CAM five days earlier, when the embryo was eight days old. Embryo on right is a 13-day-old control embryo. C. Microscopic section of a biopsy of Hodgkin's disease which produced edema of the chick embryo, and D. appearance of this tissue three days after implantation on the CAM. The cells in the implant have become pycnotic, and many have disappeared. The epithelium of the CAM is eroded, except for small islands of epithelial cells. E. Biopsy of a rapidly growing, anaplastic carcinoma of the lung, and F. appearance of cells ten days after explantation to the CAM. Nodules composed of tumor cells are seen with considerable vascular reaction around them. Mitotic figures were found in the tumor.

other types of tissues, there appears to be a specific and labile substance in lymphatic tissue which induces a transient edematous reaction of the CAM.

Chick Embryo Edema. The majority of human tissues explanted to the

CAM had no gross influence on the development of the chick embryo. One type of neoplastic tissue, obtained from patients with Hodgkin's disease, produced a generalized edema of the chick embryo in about 60 per cent of

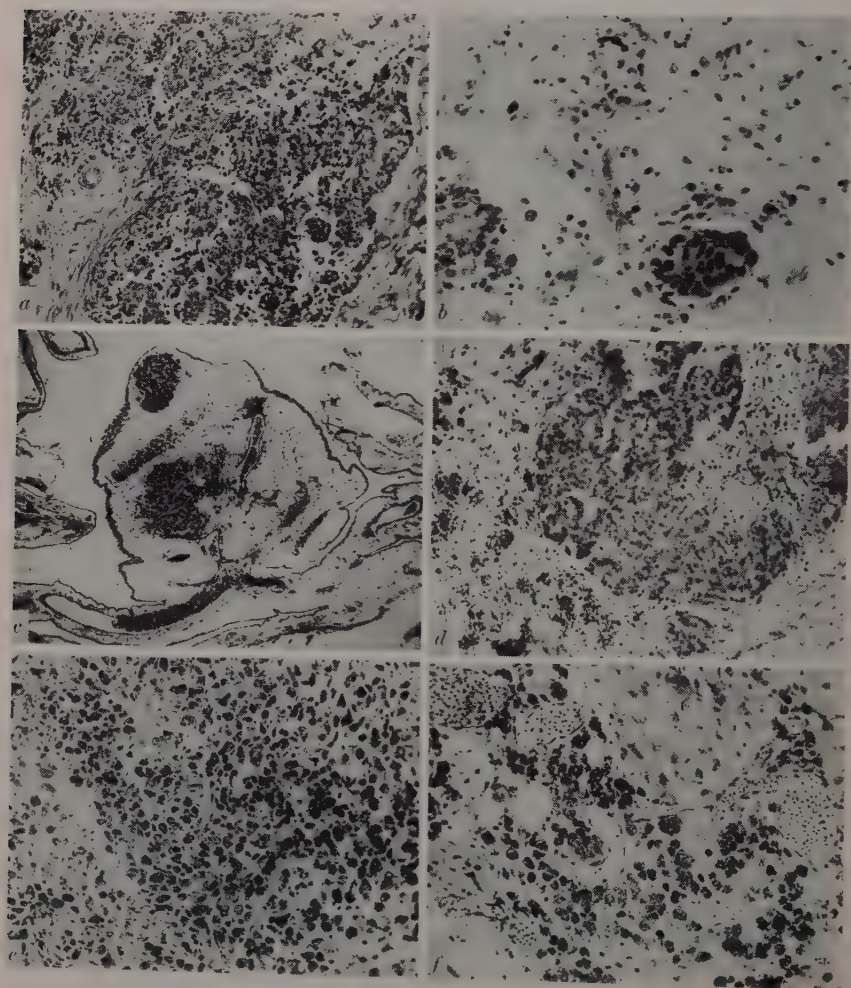


FIGURE 6. Human tumors explanted to the CAM of the chick embryo. A. Biopsy of an epidermoid carcinoma of the lung, and B. this tumor ten days after explantation to the CAM. The tumor cells are plugging a blood vessel, and the cells appear to be in good condition. C. Anaplastic carcinoma of the lung growing on the CAM of the 16-day embryo; the tumor is surrounded by large blood vessels. D. This tumor, under higher magnification, showing tumor cells apparently in good condition. E. Rapidly growing melanoma from an eight-month-old child, and F. the appearance of the CAM ten days after explantation of this tumor. Pigmented cells have infiltrated the membrane, and a rich blood supply is present. These cells may be melanoma cells, but it is possible that they are chick macrophages which have ingested the human melanin.

the biopsies tested. Small fragments of tissue, one to two mm. in diameter, placed on the CAM of the eight-day embryo, caused the maximum effect on the embryo about three days later. At this time, the embryos appeared to be developing normally, but they were distended by a clear fluid which pro-

duces a generalized edema (FIGURE 5b). Necrotic patches occasionally were seen in the liver. This syndrome was sometimes fatal, but the embryo could apparently survive this effect, and the edema had subsided seven to ten days after the tissue was inoculated. The surviving embryos, however, were smaller than normal. This edema-producing factor was not transmissible from embryo to embryo. The active material survived in the freezing compartment of the refrigerator for two months. Our present interpretation is that the factor producing the edema of the chick embryo is a chemical substance present in active concentrations in some specimens of Hodgkin's disease tissue. Whether this factor is directly associated with Hodgkin's disease, or due to the accumulation of certain cell types in the Hodgkin's disease tissue, is not known.

Failure of Lymphomas or Leukemia to survive on the CAM. In no instance did Hodgkin's disease tissue, lymphosarcoma or leukemia show any sign of active growth on the CAM, and most of the human cells were pycnotic or lysed within three to four days. The lymphomatous tissue, however, penetrated through the epithelial surface of the CAM, and in many instances the chick tissue produced a cellular reaction and blood vessels invaded the human tissue (FIGURES 5c, d). Human lymphomatous tissue thus showed a result similar to that of mouse leukemia, in its failure to grow on the CAM.

Growth of Human Carcinoma. The transplantation of human carcinoma has been sporadically successful. For example, if ten eggs were implanted with a fragment of carcinoma tissue, three may sometimes show evidence of survival of the tumor on the CAM by an enlarged nodule at the site of implantation, and the presence of human tumor cells on microscopic examination. FIGURES 5e, f and 6a-f are examples of human tumor cells surviving, and possibly multiplying, on the CAM, although in no instances could these tumors be transplanted successfully to the CAM of other eggs.

Conclusion

In this summary of the results of tumor transplantation to the chick embryo, several interesting applications of this technique may be suggested. The biological behavior of various tumors can be examined in this somewhat constant and indifferent environment. Some tumors may show unusual behavior, such as special growth characteristics or nutritional requirements which may indicate properties distinguishing them from other tumors or normal tissues. Some tumors may produce specific lesions in the developing chick embryo, such as is conspicuously evident with the Rous sarcoma, leucosis RPL-12 and Hodgkin's disease tissue, and this will be the basis for analyzing their special properties. Since some tumors can be carried by continuous passage in the chick embryo, it is possible to obtain a pure strain of tumor cells after several egg passages; the tumor cells by this time presumably will have outgrown their original stroma. This procedure may be useful for immunological studies.

Different types of tumors, including tumors from different species, can be grown simultaneously, and their biological behavior studied in this situation. Mouse and chicken tumors, growing in the egg, may be used to assay the

tumor-inhibiting activity of various agents. With the increase in knowledge of the development and metabolism of the chick embryo, important observations may result from the introduction of tumors into the embryo's environment.

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EFFECTS OF MOUSE TUMOR TRANSPLANTATION ON THE NERVOUS SYSTEM

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Mammalian tumors were first grown successfully on the chorio-allantoic membrane of the chick embryo by Murphy.⁹ The recent development of this technique has been surveyed by Karnofsky, *et al.*⁷ The first intra-embryonic transplants of normal mouse tissue were performed by Rawles.¹⁰ Bueker² has combined both techniques: he implanted mouse tumor in the body wall of three-day embryos. This experiment was performed in connection with certain neuro-embryological problems. Previous experiments had shown that the differentiation of primary sensory and motor systems in amphibia and in chick embryos is to a large extent under the control of extrinsic factors.⁴ The effect exerted by the periphery is not a species-specific; transplantations in Amphibia (Harrison,⁶ Detwiler³) had proved the capacity of nerve fibers to invade heteroplastic structures. The same compatibility was demonstrated by Bueker¹ to exist between nerve fibers of the chick embryo and the peripheral field provided by the guinea hen embryo.

By transplanting mouse tumors intra-embryonically, Bueker wanted to test the capacity of nerve fibers to invade tissue of a different class. In addition, it was his intention to confront the nerve fibers with a homogeneous tissue, rather than with a set of heterogeneous and complex structures, as represented by a limb. He performed transplantations of three different tumors: mouse adeno-carcinoma which completely failed to grow, fowl Rous sarcoma which caused extensive hemorrhage and had no effect on the nerve structures, and mouse sarcoma 180, which grew vigorously and was invaded by nerve fibers of the host. The attention was then focused on sarcoma 180.

The characteristics of tumor growth and the response of the spinal nerve centers were investigated in embryos between six and nine days of incubation, that is, between three and six days after the implantation of the tumor. In this short lapse of time, the tumor had infiltrated the somatopleure of the body wall of the embryo and bulged as a conspicuous mass in the coelomic cavity. The mesonephros was partly invaded and destroyed. In eight- to nine-day embryos, nerve bundles were found inside the tumor. The sensory ganglia on the side and at the level of the tumor were considerably enlarged in comparison with the contralateral ganglia. The size increase was 33 per cent on the average. The motor column at the same level was, on the contrary, hypoplastic. The increase in size of the sensory ganglia and the coincident reduction of the motor column were taken as evidence for the sensory nature of the nerve fibers in the tumor. The author concluded that histochemical properties of the tumor favored the branching of sensory nerve fibers, whereas somatic motor fibers were refractory to these agents.

Three important facts were thus established: (1) the sensory ganglia are responsive not only to tissues of the same class, but also to structures of a

different class; (2) the effect of sarcoma tissue on nerve fibers is selective, as proved by the admission of sensory fibers and the lack of response of somatic motor fibers; and (3) the nerve fibers act as mediators of this effect on the respective nerve centers, the spinal ganglia.

We have repeated the experiment on a large scale, using mouse sarcomas 180 and 37.⁸ The investigation was extended to the entire developmental period, and the silver impregnation technique of Cajal-De Castro was employed. The use of this technique permitted a more penetrating analysis of the relations of nerve fibers and neoplastic cells and it gave more precise information on the pattern of nerve growth inside the tumor at different developmental stages. The results of Bueker were of particular interest to us in connection with our previous study of the development of the spinal ganglia. This investigation had demonstrated in the spinal ganglia two classes of cells, which differ from each other in their developmental pattern, their affinity for silver and their topographic position.⁵ It was of interest to determine whether both classes or only one would supply nerves to the tumor.

Our investigation confirmed the findings of Bueker of a selective invasion of the tumor by sensory but not by motor fibers. A careful study of the hyperplastic spinal ganglia led to the conclusion that only the late differentiating medio-dorsal sensory neurons were involved, whereas the early differentiating ventro-lateral cells did not contribute nerves to the tumor. The overall hyperplasia in volume reached a maximum of 250 per cent in the second week of development. Another source of nerve supply to the tumor was identified in the sympathetic ganglia; their contribution of nerves to the tumor seems to be even larger than that of the sensory ganglia if one uses the degree of hyperplasia as a criterion. Sympathetic ganglia adjacent to the tumor underwent a hyperplasia which reached a maximum of 600 per cent. In both sympathetic and spinal ganglia, the overall increase in volume is the result of at least three different factors: increase in cell number, increase in the size of individual cells, and acceleration in the processes of cell differentiation.

The ingrowth of nerve fibers into the tumor begins on the sixth day of development. In the preceding stages (four to six days), nerve fibers grow out from the somato-motor column and from the early differentiating sensory neurons. They fail to invade the tumor, although the latter is readily accessible to both.¹⁸ From the sixth day on, nerves enter the tumor in increasing numbers. Sensory and sympathetic components can be easily traced to their origin (FIGURE 1); in the tumor, the fibers are intermingled and not distinguishable from each other (FIGURE 7). In the majority of the cases, the nerve bundles end abruptly without establishing contact with individual tumor cells. They branch in an irregular fashion and give origin to an extremely dense net of nerve fibers. No embryonic or adult tissue shows a nerve distribution of comparable density.

As a result of this investigation, it was concluded that sarcomas 180 and 37 release a growth promoting agent which stimulates selectively the growth of late-differentiating sensory cells and of sympathetic ganglia. Since the

publication of our first results, a large number of additional intra-embryonic transplants were performed, including 95 transplants of sarcoma 180, and 225 of sarcoma 37. An effort was made to raise the embryos to advanced stages. However, only a few embryos older than 14 days were recovered. The high rate of mortality of embryos in later developmental

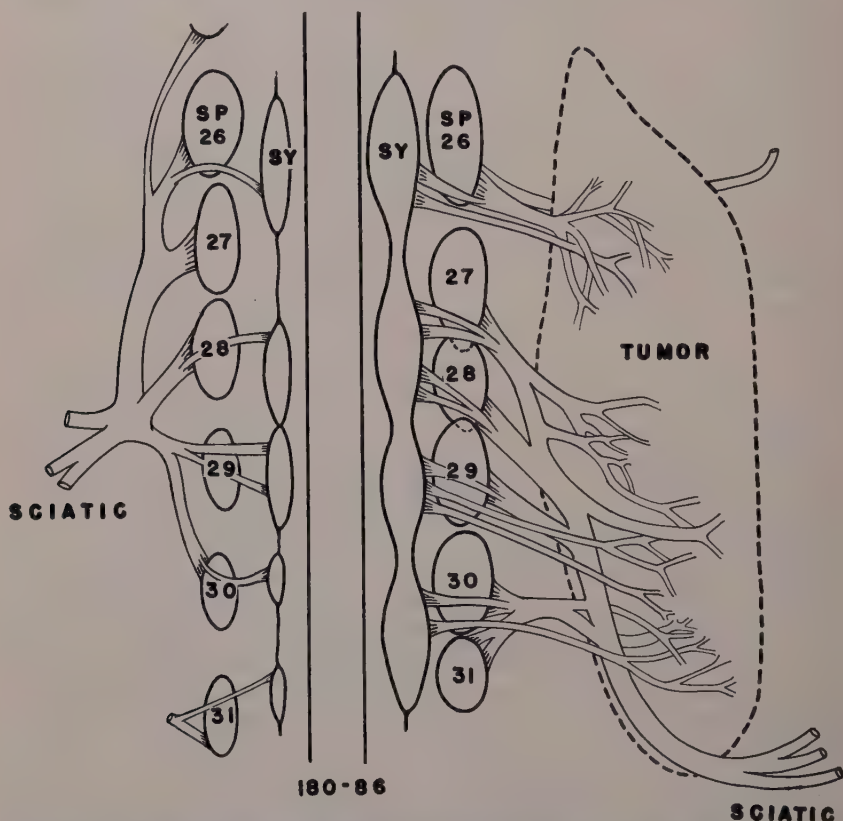


FIGURE 1. Fifteen-day embryo. Semi-diagrammatic reconstruction of the distribution of nerve fibers in the tumor (sarcoma 180). SP, spinal ganglia; SY, sympathetic chain ganglia. (From Levi-Montalcini and Hamburger, 1951, *J. Exp. Zool.* 116: FIGURE 3.)

stages seems to be correlated with toxic effects of the tumors, which reached a very large size in the third week of incubation.

It was found that, in addition to nerve centers which are directly connected with the tumor by nerve fibers, paravertebral and prevertebral ganglia which had no fiber connections with the tumor were also hyperplastic. This effect is particularly striking in the thoracic segments of the prevertebral sympathetic chain of 10- to 16-day embryos. Where one finds normally a pair of ganglia at the two sides of the aorta, and two slender strands of nerve fibers connecting these ganglia with the adrenal and hypogastric plexuses, one finds now large ganglionic complexes bulging laterally to the

aorta (FIGURES 4, 9, and 10). Thick nerve bundles emerge from their caudal ends and give origin to the prevertebral chains. They are many times larger than those in control embryos. The celiac plexus which, under normal conditions, is rather rudimentary until the tenth day of incubation, is already conspicuous in experimental animals on the tenth day of incubation. It forms a dense meshwork of fibers around the celiac artery, which increases in density during the following days. The celiac plexus is the main source of nerve supply to the abdominal viscera. The pattern of nerve ingrowth into the viscera of normal embryos and embryos carrying tumor transplants will be described below.

In addition to the ganglia adjacent to the aorta, one finds in normal embryos, at the level of the adrenal primordia, groups of nerve cells which are scattered among the strands of cortical cells. Under the impact of the tumor, these clusters of cells are now transformed into large ganglionic masses. An equally striking enlargement was found to occur in the hypogastric ganglia. As a result, the entire prevertebral thoracic and abdominal chain is transformed in some cases into a massive cellular column. Moreover, one occasionally finds ganglionic masses stemming from this column which project into the fringe of the tumor (FIGURE 8); they have no equivalent in normal embryos.

The intrinsic ganglia of the stomach and intestine (Meissner and Auerbach plexuses) were entirely unaffected.

The overgrowth of the ganglia which had no fiber connections with the tumor suggested that this effect on the sympathetic system was mediated through the blood circulation. To test this hypothesis, the tumor was transplanted to the extra-embryonic coelomic cavity, near the presumptive yolk stalk of three-day chick embryos. When it was realized that some sympathetic nerve fibers could still reach the periphery of the tumor, the grafts were made to the allantoic vesicle of four-day chick embryos. A total of 260 transplants were performed: 138 in the yolk stalk and 122 on the allantoic vesicle. The embryos were fixed between seven and 17 days of incubation. They were impregnated with silver and sectioned serially. The tumor grafts were recovered in every case and studied histologically.

Extra-embryonic Transplants

1. *Transplants Near the Presumptive Yolk Stalk.* The tumor became encapsulated in the yolk stalk and bulged laterally in the extra-embryonic coelomic cavity (FIGURE 2). Toward the tenth day of incubation, it had, in some cases, reached the size of a pea. Generally, however, it was of a smaller size. All embryos of this series were fixed between seven and ten days. Sections showed that a few nerves emerging from the extrinsic intestinal ganglia (Remak nerve) had reached the periphery of the tumor and branched in its peripheral parts.

2. *Transplants onto the Allantoic Vesicle.* At four days, the allantois bulges into the extra-embryonic coelomic cavity as a small pear-shaped vesicle. It has not yet come in contact with the chorion membrane. One or two fragments of sarcoma 37 or 180 were grafted onto the allantois

after perforation of the chorion. A considerable number of transplants became established and grew vigorously during the days following the transplantation. At ten days, the size of the grafts was about the same as in the

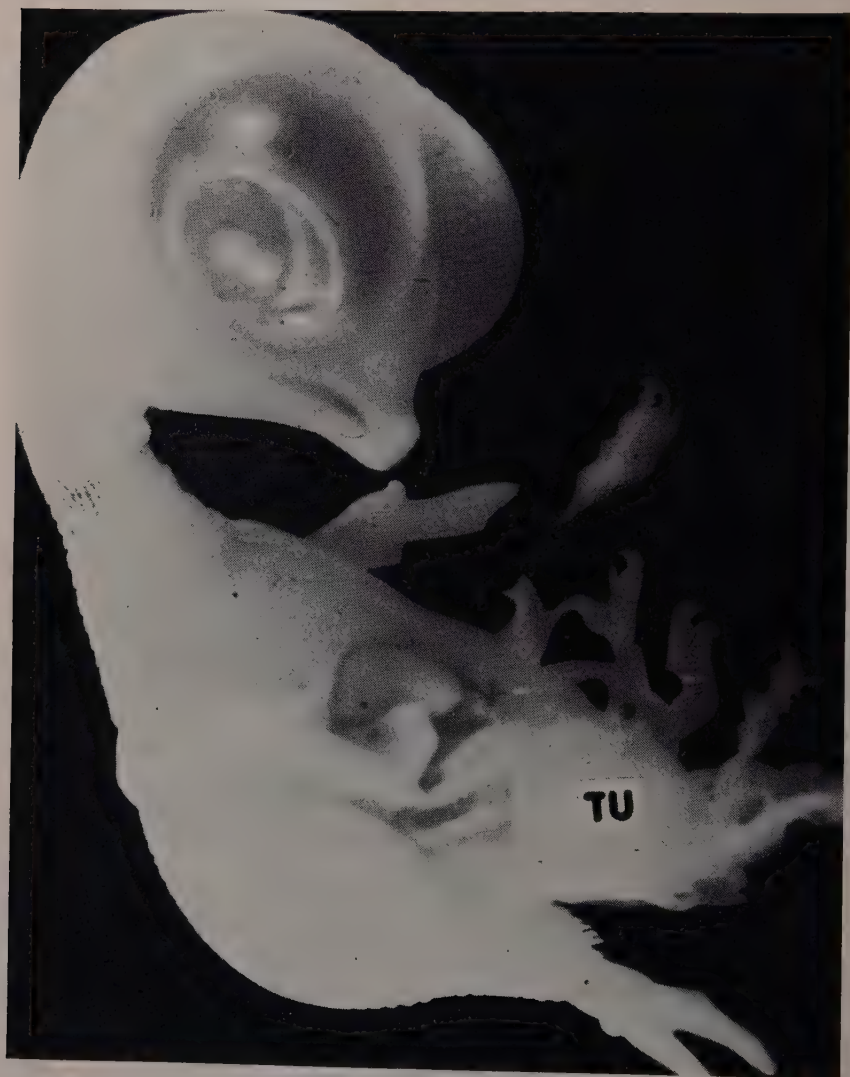


FIGURE 2. Ten-day embryo. Tu, tumor (sarcoma 37) embedded in the yolk stalk.

previous experiments. In the following days, they reached the size of a bean (FIGURE 3) and they were even larger in those embryos which survived until the 16th day of incubation. Up to 12 days, the embryos showed no sign of impairment. From then on, they appeared to be significantly smaller than control embryos: the spleen was enlarged and the liver deeply suffused

with bile. Similar and other noxic effects had been described as a result of serial transplantation of mouse adeno-carcinoma to the yolk sac by Taylor and Carmichael.¹¹ The rate of mortality was highly increased after the 14th

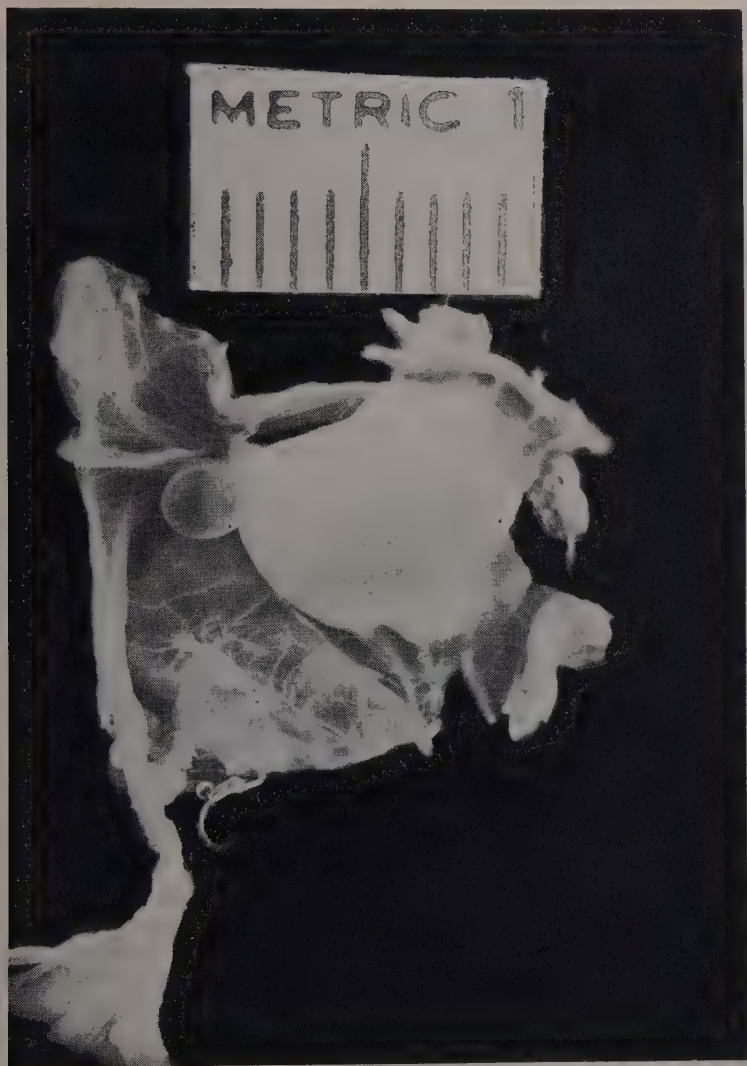


FIGURE 3. Tumor (sarcoma 37) growing on the allantoic membrane of a 14-day embryo.

day, as in the intra-embryonic transplants, and no embryo survived the 17th day of incubation. In many cases, the tumor infiltrated the blood vessels of the chorio-allantoic membrane, resulting in lethal hemorrhages.

In no cases of transplants to the allantoic membrane were nerve fibers

found in the tumor. In the majority of extra-embryonic transplants, metastatic tumor cells were found in the host. Small neoplastic nodules were scattered in the mesenteries or became established at the fringe of the thoracic or abdominal organs. The mesonephroi were the preferential site of their formation. In a few cases, metastases completely failed to occur. If the metastatic nodules happened to be within reach of visceral nerve fibers, they were invaded by them. There was no correlation, however, between the hyperplastic condition of the visceral ganglia, as described below, and the nerve connections with tumor metastases. In cases in which metastases did not occur, the effect of the extra-embryonic tumor was the same as in the cases with multiple metastases. Therefore, the occasional ingrowth of nerves into the metastatic nodules was not instrumental in the overall tumor effect. The same reasoning holds for the nerve ingrowth into the peripheral areas of yolk-sac transplants. The yolk transplants and the allantoic transplants, therefore, will be considered together in the following discussion.

The effects of extra-embryonic transplants on the sympathetic paravertebral and prevertebral chain ganglia were qualitatively identical with intra-embryonic transplants of the tumor (FIGURE 4). In all cases, the whole sympathetic system was highly hyperplastic. A total of 35 embryos were investigated in detail. Area measurements were made of the prevertebral sympathetic chain ganglia of the thoracic and abdominal level between the caudal end of the brachial spinal cord and the rostral end of the lumbo-sacral cord. Camera lucida drawings of the contours of the ganglia were made in every other section, and the areas measured with the planimeter; altogether, nine experimental cases and six control embryos were used. The results are represented in FIGURE 5. A hyperplasia of the sympathetic system was already detectable at the end of the seventh day of incubation, whereas the intra-embryonic transplants had no effect in corresponding stages. In the following stages, between eight and 12 days, the effects of intra-embryonic and extra-embryonic transplants are similar in every respect. After the 12th day, the intra-embryonic transplants have a more conspicuous effect than the extra-embryonic transplants. In these late developmental stages, the intra-embryonic tumors have generally reached a larger size than the extra-embryonic transplants, and a relation between the size of the tumor and the amount of the hyperplasia of the sympathetic system suggests itself. No quantitative measurements were made, however.

FIGURE 5 shows that the effect of both intra- and extra-embryonic tumors are progressive and that the effect of the former reaches a maximum of over 800 per cent in 16-day embryos.

Peripheral Distribution of Visceral Nerve Fibers

The next question to be discussed is the distribution of the nerve fibers which emerge from these hyperplastic ganglia.

A complete series of silver-impregnated normal embryos was available for comparison. It may suffice to mention here that, in normal embryos, from the ninth day on, a very small number of visceral nerve fibers can be traced along the main blood vessels into the spleen, ovary, testis, liver, meta-

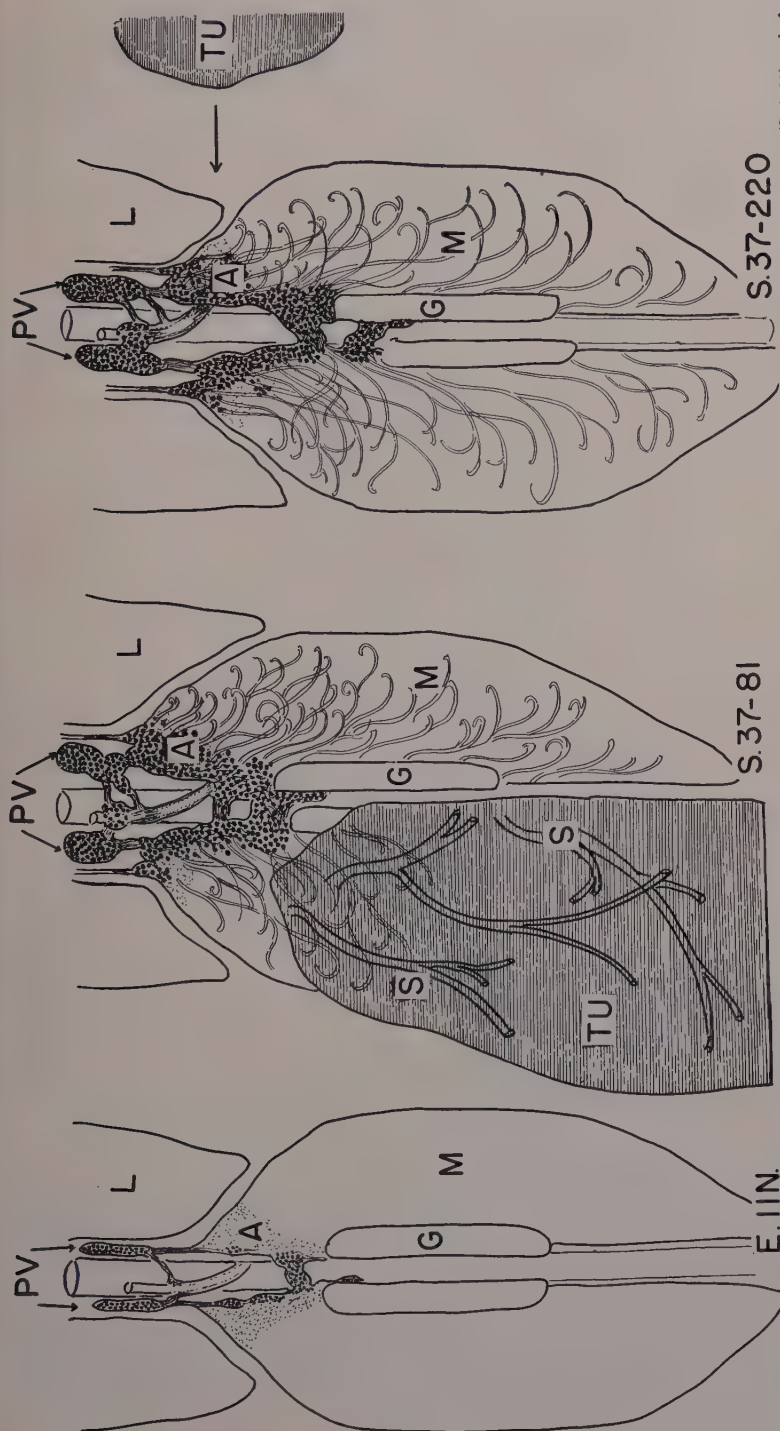


FIGURE 4. Semi-diagrammatic reconstruction of a normal 11-day embryo, (E 11n), of an 11-day embryo carrying an intra-embryonic transplant of mouse sarcoma (S 37-81) and of an 11-day embryo with transplant of sarcoma 37 on the allantoic vesicle (S 37-220). Notice the hyperplastic growth of the prevertebral chain ganglia in embryos carrying tumor transplants. Visceral nerve fibers from these ganglia invade the nearby mesonephroi. A, adrenal; G, gonad; L, lung; M, mesonephros; PV, prevertebral ganglia; S, sensory nerves; TU, tumor.

nephros, thyroid gland and pancreas. In the mesonephros, a visceral innervation was not observed. In embryos with tumor grafts, all these organs were swamped with nerve fibers from the very beginning of their differentiation. Nerves from the nearby paravertebral and prevertebral ganglia enter the rostral edge of the mesonephros at the end of the seventh day of incubation and grow along the mesonephric tubules, following a rostro-caudal course. In the following days, the number of these nerve bundles

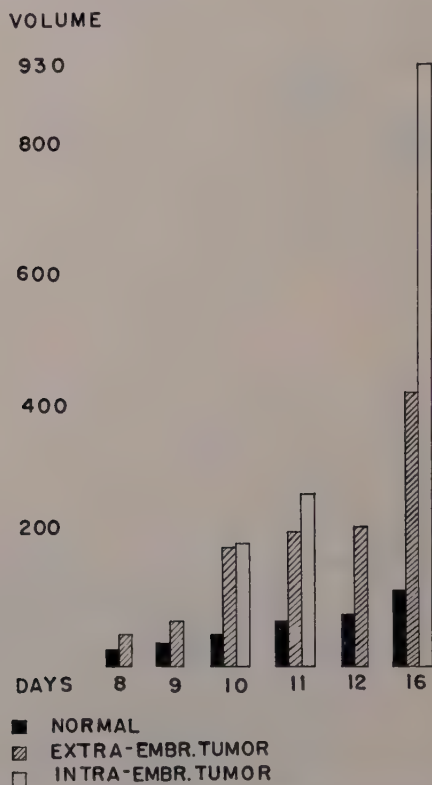


FIGURE 5. Volume increase (in arbitrary units) of the prevertebral thoraco-abdominal chain ganglia in control and experimental embryos, between 8 and 16 days of incubation.

increases rapidly and the entire organ is literally flooded with visceral nerve fibers which can be traced easily to the enlarged ganglia (FIGURE 4). A maximal density of nerve fibers was observed in those cases in which the area measurements had shown a maximal hyperplasia of the sympathetic ganglia. The nerves do not establish connections with the mesonephric tubules; they follow the blood vessels and wrap themselves around them (FIGURES 15, 16).

A similar pattern of nerve distribution was observed in the other organs mentioned above. The spleen, liver, ovary (FIGURES 13, 14), testis, metanephros, pancreas, ultimo-branchial bodies, and thyroid gland (FIGURES 17,

18) are all invaded by nerve fibers as soon as they start to differentiate. In every organ, the nerves enter with the blood vessels. Whereas the pattern of invasion is consistently the same in all cases, the density of nerve fibers

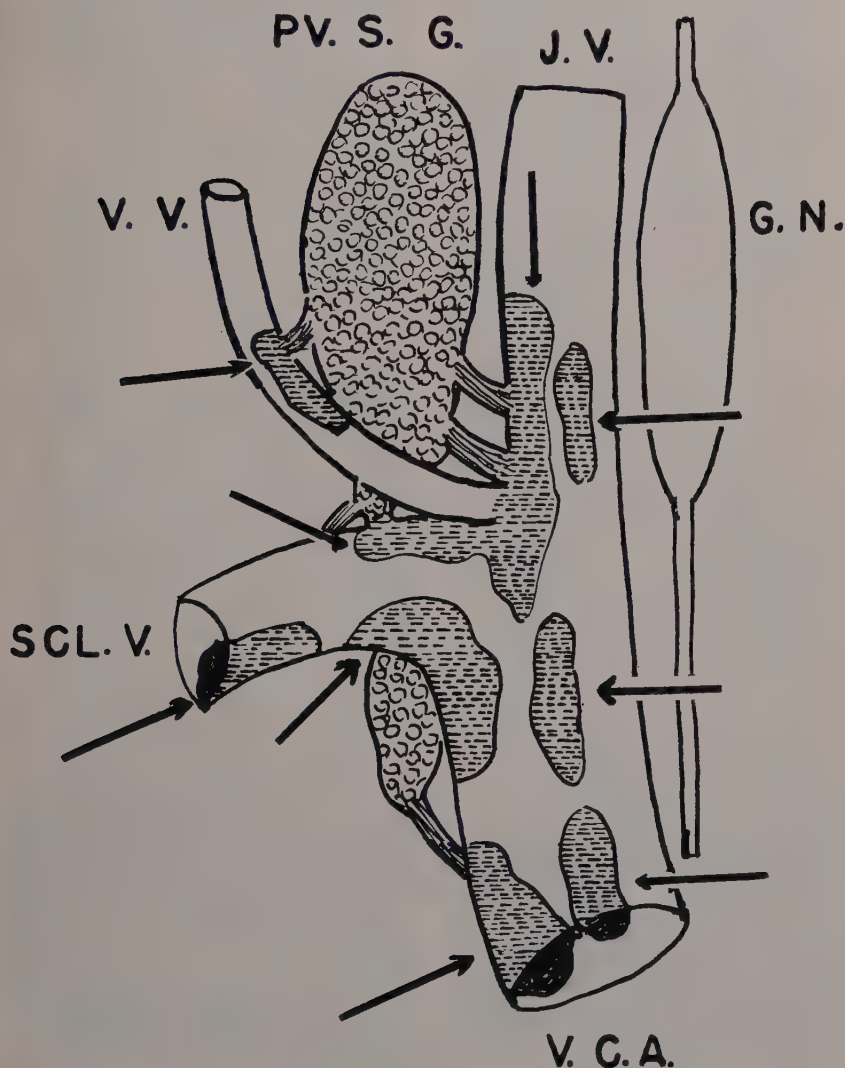


FIGURE 6. Sixteen-day embryo with intra-embryonic tumor (sarcoma 180). Ingrowth of sympathetic nerve fibers into the Jugular, Vertebral, Subclavian, Anterior Caval Veins. GN, Ganglion Nodosum; JV, Jugular Vein; Pv.SG, Paravertebral Sympathetic Ganglion; SCL.V, Subclavian Vein; VCA, Anterior Caval Vein; VV, Vertebral Vein. Arrows point to nerve agglomerations.

shows a high degree of variation in the different embryos. The amount of nerve fibers in the viscera is proportional to the degree of hyperplasia of the visceral ganglia. This, in turn, is dependent on the size of the tumor, as mentioned above.

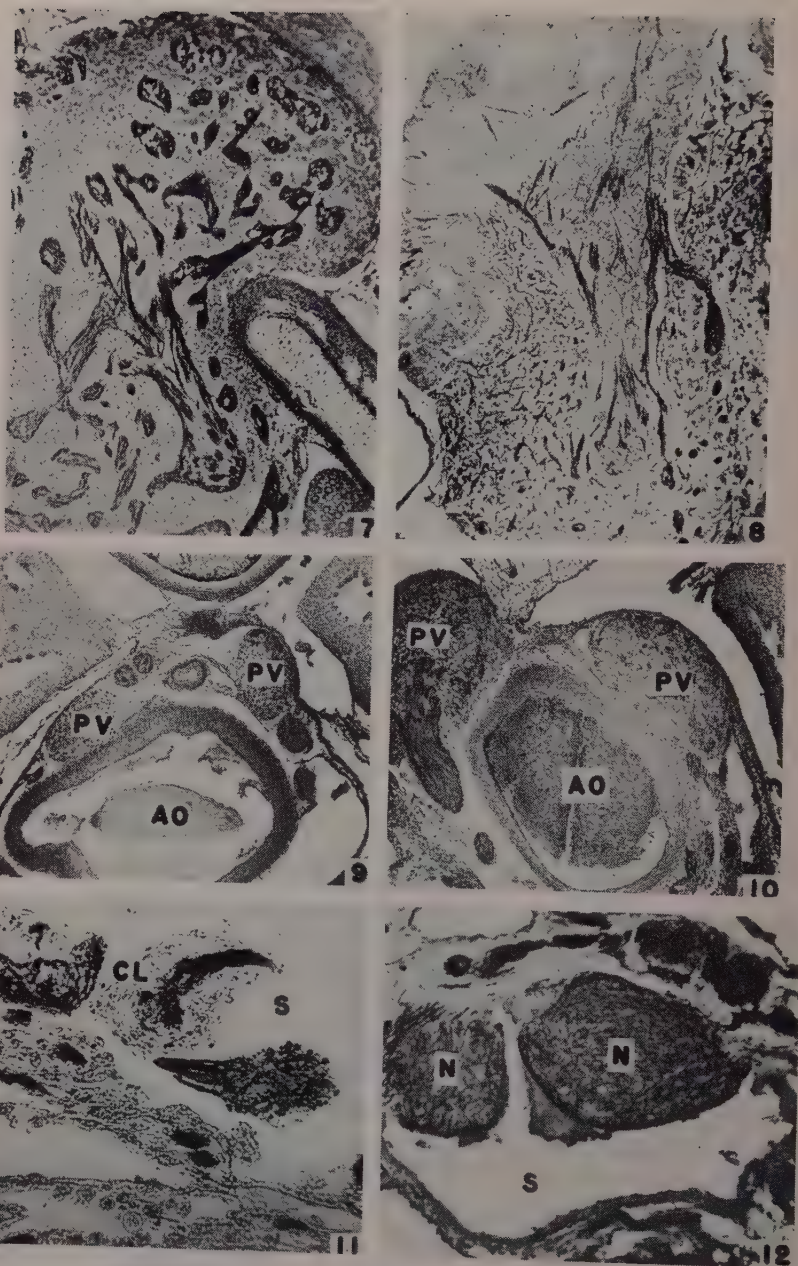


PLATE I*

FIGURE 7. Intra-embryonic tumor (sarcoma 180) in 11½-day embryo. Section through part of the tumor to show density and irregular branching of nerve bundles. (From Levi-Montalcini and Hamburger, 1951, *J. Exp. Zool.* 116: FIGURE 14.)

FIGURE 8. Nеоformation of sympathetic ganglion projecting into the fringe of the tumor (sarcoma 180) of an 11-day embryo. Nerve fibers from the ganglionic mass invade the tumor.

FIGURE 9. Prevertebral sympathetic ganglia (PV) on both sides of the aorta (AO) in a normal 16-day embryo.

FIGURE 10. The same ganglia in a 16-day embryo with an intra-embryonic tumor (sarcoma 180). AO; Aorta; PV, Prevertebral sympathetic ganglia. Same magnification as FIGURE 9.

FIGURE 11. Twelve-day embryo with graft of sarcoma 180 on the allantoic membrane. Visceral nerve bundles have perforated the intima and end freely inside the subcardinal vein (S). Blood clots surround the nerve fibers (CL).

FIGURE 12. Sixteen-day embryo with intra-embryonic tumor (sarcoma 180). Two large nerve agglomerations of visceral nerve fibers (N) bulge into the subclavian vein (S).

* All photographs are of silver-impregnated material (De Castro's modification of Cajal's method); all are unretouched; white demarcation line added to FIGURE 8.

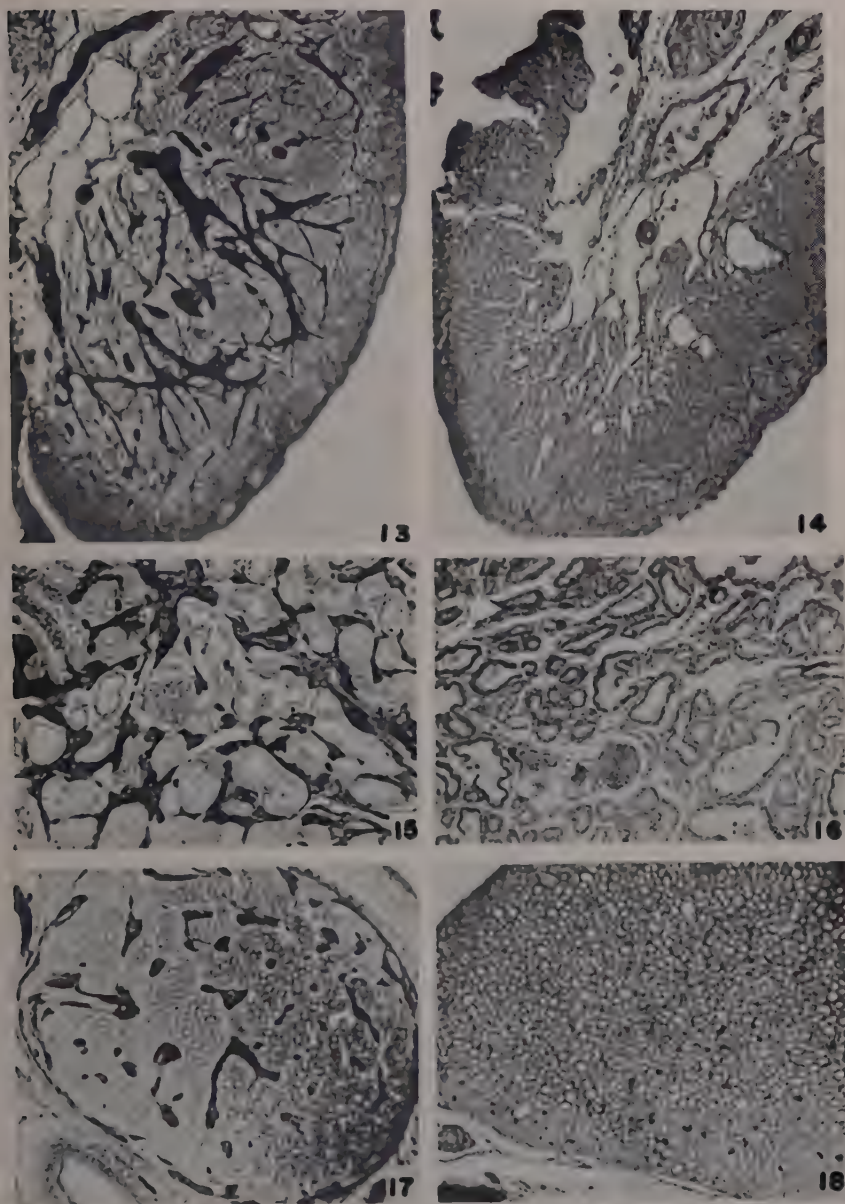


PLATE II

FIGURE 13. Branching of visceral nerve fibers in the ovary of a 16-day embryo with an intra-embryonic tumor (sarcoma 180).

FIGURE 14. Ovary of a control embryo of 16 days.

FIGURE 15. Visceral nerve fibers in the mesonephros of a 16 day embryo with an intra-embryonic tumor. The nerve fibers wrap themselves around the blood vessels.

FIGURE 16. Mesonephros of a control embryo of 16 days.

FIGURE 17. Thyroid gland of a 16-day embryo with an intra-embryonic tumor (sarcoma 180). The nerves enter the gland with the blood vessels.

FIGURE 18. Thyroid gland of a control embryo of 16 days.

A very unusual relation was found between sympathetic nerve fibers and the large veins of the thoracic and abdominal level. Whereas one normally finds small numbers, at best, of fine fibers in the media, but none in the intima, the experimental cases show at 11 days the penetration of numerous visceral nerve bundles into the intima of the large veins, such as the jugular, pre-caval, subclavian, and the subcardinal (FIGURES 6, 11, and 12). At 16 days, these nerves have enlarged to a very conspicuous size and bulge into the lumen of these veins in the form of thick nerve agglomerations (FIGURE 12). In some instances, they grow to such a size that the lumen of the blood vessels becomes obliterated. Clusters of blood cells or large blood clots surround the protruding nerve fibers. The arteries and the capillaries are spared from invasion. The death of practically all embryos toward the 17th day of incubation prevented a further analysis of the fate of these aberrant fibers.

Concluding Remarks

The investigation is still in progress and we refrain from a theoretical interpretation of these results. We can state, however, that the humoral transmission of a tumor-produced agent, which affects profoundly the development of the sympathetic system, is definitely established by the extra-embryonic transplants. These results are not in conflict with the previously-advanced hypothesis^{2, 18} that nerve fibers mediate the tumor effect whenever they penetrate the tumor. We are not yet in a position to decide if we are dealing with two different modes of action or whether the same mechanism operates in both instances.

We should like to emphasize the exceptional character of the response of the visceral nervous system to the tumor. The results reveal a morphogenetic effect for which there is no parallel. The selective susceptibility of some neurons to the effect of the tumor and the absence of species specificity may suggest an analogy between this and hormonal effects. One aspect of the effect of the tumor does not fit in this picture, however. The tumor promotes an excessive growth of the sympathetic system with total disregard of the requirements of the whole organism. In fact, it upsets its harmonious development to such an extent that it causes profound changes in the sequence of developmental processes. Whereas, under normal conditions, proliferation and differentiation of the sympathetic ganglia are held within limits and kept in pace with the development of other structures, we find that under the impact of the tumor the sympathetic system overtakes other systems. The increase in the size of the sympathetic chain ganglia which, in one particular case, amounts to more than eight times the normal size, is even more impressive if one realizes that the embryos were considerably smaller than normal embryos, due to toxic tumor effects. The latest stages investigated are 16- and 17-day embryos, and there is no indication that the hyperplastic process has come to an end at that time.

The accommodation of an enormous number of nerve fibers in the viscera is equally intriguing. It indicates another severe infraction of the laws which govern the mechanics of development. Under normal conditions,

nerve fibers are admitted to organs according to a rigid time schedule, and the quantity of the entering fibers is strictly limited; each organ has a characteristic density, and regulative mechanisms are in operation to prevent hyper-neurotization. In the present instance, all barriers seem to have broken down, and the organs surrender to the invading fibers. It remains to be seen whether some properties of the nerve fibers are changed, such as their invasiveness or growth potential, or whether the organs themselves are directly affected by the tumor. An even more striking instance of the abnormal behavior of visceral nerve fibers is represented by their perforation of the intima of the veins, and the formation of nerve agglomerations in the lumen of these vessels. Nothing can be said concerning the nature of the agent and our research is now heading in this direction.

Two other questions arise in connection with the present investigation:

1. Are the nerve cell bodies or the nerve fibers the immediate target of the tumor agent?

2. What is the source of origin of the greatly enlarged and the newly-formed sympathetic ganglia? In view of their enormous volume and relatively late formation of additional ganglia (after the seventh day), it seems difficult to accept their derivation from the neural crest. In this connection, the contention of Tello¹²⁻¹⁴ that the sympathetic cells are mesodermal derivatives and have a common origin with the endothelial cells of thoracic blood vessels deserves serious consideration.

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Discussion of the Paper

DR. DAVID A. KARNOFSKY (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): Dr. Levi-Montalcini has found that the embryos transplanted with Sarcoma 180 die about the 16th day of development, presumably due to the growth of the tumor. We have noted that the chick embryo is relatively resistant to the nitrogen mustards. The injection of a dose of methyl-bis (B-chloroethyl) amine hydrochloride, about $\frac{1}{5}$ of that lethal to the chick embryo, is destructive to the sarcoma 180 previously transplanted to the chorio-allantoic membrane. (Ref.: Approaches to Tumor Chemotherapy, A. A. A. S., pp. 293, 1947). After the sarcoma 180 has induced the enlargement of the ganglia in the chick embryo, it may be possible to save the embryo by destroying the tumor with nitrogen mustard, and then see if the overgrowth of nervous tissue persists or regresses.

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